

Beyond the neutron bomb

PRESIDENT Carter's decision to defer further work on the neutron bomb is being variously interpreted as a fine gesture towards unblocking log-jams in disarmament, a surrender to Soviet pressure or yet another sign of vacillation in the White House. In fact, his decision owes as much as anything to European politics, and vacillation is as much on the eastern as the western side of the Atlantic.

The neutron bomb, its history going back to the 'clean' bombs much talked of in the early 1960s, is a weapon that delivers a large dose of immediate radiation but a relatively small amount of blast and long-term radioactivity. It is thus of most use in battlefield contexts where the requirement is to eliminate the opposition without defiling the territory they occupy. If ever there were to be a battle between NATO and Warsaw Pact forces, continental Europe, and more specifically Germany, could be the only location for the foreseeable future.

The United States, seeing a remorseless build-up of forces in Eastern Europe, has been trying to get West Germany and, to a lesser extent the United Kingdom, to show some enthusiasm for the weapon, as it could hardly be sold to

the American public on domestic needs. The enthusiasm has been slow in forthcoming—not from the military but from politicians who found it too vexed an issue for an unequivocal endorsement. So, without total support from his NATO allies, Mr Carter has cut his losses.

Even though this interpretation portrays the President as less of a bringer of peace to all the world than do some other interpretations, there is still an opportunity, in the deferment and probable cancellation of the neutron bomb, for the cause of disarmament to profit. The neutron bomb was cause for concern on two counts: it was yet another example of the vertical proliferation—more and more sophisticated weaponry—that non-nuclear countries find so offensive; and it provided a stepping stone across the gap between conventional and nuclear warfare. We have cause to be grateful that these two concerns have been allayed. We also have cause to be grateful that Mr Carter has broken away from conventional doctrine that new weapons must be used as bargaining chips in negotiations. But now the onus is on the Soviet Union to respond to this unilateral measure with some imaginative gesture of its own. □

Classified information

ON the right is one advertisement that has never appeared in this, or in any other form in *Nature*. The top people in Britain's scientific administration are appointed after a discreet process of sounding out feeling—one almost expects a puff of Pontifical smoke to emerge over Whitehall after the selection has been made. The high calibre of the holders of the posts could, however, be used as clear evidence that the system works.

So it does at present. But there is a lot to be said for casting the net wider. There are many excellent scientists working in unspectacular backwaters who might perform these jobs very well. There are many younger scientists whose interest in science policy should be stimulated in the same way that the United States government brings people to Washington in their thirties. There is a vast number of British scientists working abroad who might bring some very different perspectives to these posts.

It would still be open to the government to choose someone nominated from within the system, of course. But a lot might be learnt by making the selection process a little more accessible.

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The above are term appointments; vacancies usually occur at the rate of one or two per year. Since the holders of these posts exercise considerable control over science policy and its interaction with other affairs of state, Her Majesty's Government are particularly concerned to hear from as wide a range of applicants as possible, well before any particular vacancy falls due. Candidates from the academic and educational world, the civil service, industry, commerce, the media and the professions will be equally welcome to apply. It is especially valuable to hear from expatriate Britons.

Candidates should preferably be over 35 and have experience of many different facets of science and/or technology; at least one previous position should have involved substantial administrative responsibility. There will be considerable travel, some of it overseas. The posts are arduous, but with adequate remuneration.

There is no set procedure for applying for these posts. Those interested are invited to write in confidence to the Lord Privy Seal, the government minister responsible for coordination of science policy, at the Civil Service Department, Whitehall, London SW1 mentioning the post(s) of interest and outlining their experience. A formal acknowledgement will be made, and candidates whose case is strong will be invited at a later date to an exploratory interview, even if at present no suitable position is vacant.

Candidates of either sex are equally encouraged to apply. Nominations, as well as applications, will also be considered.





WINDSCALE may go ahead from 15 May with the design and construction of a 1,200 tonne per year reprocessing facility for oxide nuclear fuel, if the Special Development Order (SDO) laid before the British parliament last week takes effect. The order is subject to "the negative resolution procedure"—which is to say it passes undebated unless a member of parliament opposes it. The Liberals have already done so, and a group of Labour MPs convened by Robin Cook (Edinburgh) are expected to follow suit this week. The result is that there must be a further debate on Windscale before the 15 May deadline.

"The later the better" said Leo Abse this week. Abse is one of the MPs opposed to the Windscale expansion, and he would like as long as possible before the debate "for public opinion to take effect". Friends of the Earth have a demonstration planned for Trafalgar Square on 29 April, and there is some conflict between those who would like the debate before the demonstration and those who would

Windscale to be debated again

like it after. Abse will see Michael Foot, Leader of the House, this week to impress on him that the issue "mustn't have the appearance of being manipulated"—as he clearly believes it has. "They laid the order the day we got back from the Easter recess" he said. "Government are anxious to get the matter through before public opinion builds up".

Frank Hooley, another MP opposed to Windscale, believes that the SDO will be carried whenever the debate takes place. "But I think opinion will move the other way—if only because of the costs". Effective safeguards, monitoring, and disposal of the waste will ultimately be seen to make the plant uneconomic, Hooley believes.

Another factor in the debate will undoubtedly be the Nuclear Non Proliferation Act signed by President Carter last month. The Act threatened to cut off all nuclear fuel supplies to the EEC unless the community agreed to renegotiate the supply treaty with the US. This 20-year-old treaty allows Europe to re-export the fuel or its products within its boundaries; so West Germany, for example, would be free to export waste to be reprocessed at Windscale or Cap la Hague in France. The treaty with Japan, on the other hand, allows no movement without US agreement.

The US Senate sub-Committee on energy and non-proliferation wrote to Carter in March expressing disgust at the "mockery" made of US non-proliferation policy by the expansion of reprocessing plants in Europe and it may be now that the open disavowal of US policy expressed by British Foreign Secretary David Owen in the last Windscale debate (22 March) is beginning to have its effect.

Robert Walgate

Poland plans hydrological atlas

DEPARTMENTAL interests are complicating the optimum use of Poland's water resources, according to K. Frankowski, Director of the Wroclaw provincial water conservancy directorate. Speaking on Warsaw Television, he stated that at present, every drop of water in Poland has several owners: reservoirs at power stations belong to the Ministry of Mining and Power, and all other reservoirs to local water conservancy directorates which ultimately come under the Ministry of Agriculture. The Odra, the Vistula, and all the canals come under Shipping, while water intake and effluent plants are the responsibility of the Ministry of Administration, Local Economy and Environment.

Poland has a relatively low water supply (50 thousand million m³/year), which, moreover fluctuates considerably over the year, so that in terms of water resources, Poland ranks 22nd in Europe, comparable only to the dry steppe areas of Ukraine and to the driest regions of Spain.

At present there exists on paper an 'Outline of the longterm plan of water economy in Poland', which en-

visages the construction of reservoirs in the Carpathians with an overall capacity of 3,700 million m³, flood-control and retention projects on the Vistula, Pilica and Bug and in the Mazurian lake district, and the planting of forests to hold back the run of surface waters and prevent soil erosion. To carry out all engineering works necessary to ensure Poland's water supply after 1990 will cost at the very least 1,500 million Zloty (£750 million).

According to Frankowski, while Poland's water resources remain under divided control, each of the Ministries concerned will continue to regard water as "free wealth" coming in unlimited quantities, and the plan will continue to exist on paper only. Already the piecemeal renovation of Poland's inland waterways by different authorities has led to anomalies, such as, for example, the upper reaches can accommodate larger barges than the lower. During the last few years considerable legislation has been passed on pollution-control and water utilisation; however, with an estimated three-fold increase in the total demand for water over the period 1970-1990, urgent action, rather

than legislation, would seem to be the key. Frankowski's broadcast urged the setting up of a single responsible body to co-ordinate all measures dealing with water conservation. But on what are such decisions to be based? Although a number of development studies have been carried out (partly financed by the UN), notably the plans for the Vistula and Odra basins, the definitive hydrological atlas of Poland, described as a breakthrough work for Polish science is still not ready. This *opus*, which will present the hydrological relations between the geographical environment and the climate... contain data concerning the water balance, water diffusion from river evaporation, snowfall, the supply of rainfall to water basins, groundwater... ice on rivers and lakes... the water levels in rivers and other water basins, the transport of debris by rivers, the effects of flood levels, and low-water levels in summertime... would appear to be essential for any comprehensive planning. Yet, according to Dr Juliusz Stachy, head of the production team, data gathering for the atlas has only "just crossed the half-way mark". Dr Stachy is sure that the atlas will be ready by 1980.

Vera Rich

UNEP scheme to bring power to Pakistan's villages

ALTHOUGH some of Pakistan's urban centres are based on those of affluent societies, 75% of its population, inhabiting 45,000 villages, still live in the 5,000 year-old Moenjodaro style. All but 6,000 of the villages are without electricity.

An experiment, based on studies made a couple of years ago for the United Nations Environment Programme (UNEP), however, could be a harbinger of change and bring improvements to the quality of life in rural Pakistan. Proposed by Dr I. H. Usmani, Energy Adviser to the United Nations Centre for Natural Resources, Energy and Transport in New York, the scheme is designed to harness solar energy, wind power and village bio-mass to generate electrical power on a suitable scale for a village of 100–150 families. These generating stations, to be called Rural Energy Centres (REC), will only use commercially available, proven technological devices.

Dr Usmani, who has been both Chairman of the Pakistan Atomic Energy Commission and Chairman of the Board of Directors of the International Atomic Energy Agency (IAEA), has ceased to be a nuclear enthusiast since he joined UNEP five years ago. During a recent visit to Pakistan he discussed his REC plan in some detail with General Zia-ul-Haq, Chief Marshall Law Administrator.

The UN has agreed, in principle, to establish four RECs in Pakistan. The cost will be met from Pakistan's country programme quota and \$800,000 set aside for the purpose. Dr Usmani spent some of his visit finalising the project document for the four proposed units, one in each province of Pakistan. A fifth REC, however, may precede the UN-sponsored ones. Dr Usmani said that General Zia-ul-Haq was so pleased with the idea that he wanted one REC, financed by Pakistan's government, to be

set up as soon as possible in a village near Rawalpindi. The UN-sponsored centres may take two or more years to become operational.

The UN is already establishing three RECs in three other third world continents. One is in Sri Lanka in Asia, one in Mexico in Latin America and the third in Senegal in Africa. All three are beyond the drawing board stage. Quotations have already been received for the Sri Lanka centre. It will cost between \$300,000 and \$350,000 and will generate 100–150 KW of electrical power.

The design of individual RECs will be dependent on local geography and the local potential of renewable energy sources, especially solar, wind and water power and bio-mass. Bio-mass will be fermented to produce bio-gas and burnt to produce char and oil as solid fuel for cooking.

A typical REC will include:

- A solar-thermal device. This will use either low-grade heat to drive a freon turbine in a Rankine cycle engine

generator producing about 10 KW; or high temperature steam to drive a conventional engine.

- A panel of solar photo-voltaic cells to produce 2 KW. This would give about 8 KW per day on average.

- Two wind generators of 2–4 KW.

- 25 KW capacity diesel generators modified to work on bio-gas.

- A long-life battery of lead-calcium-antimony cells to store 150–300 KWh (e) per day.

The electrical energy obtained could be used by 100 village families to pump well water, light homes, schools and streets, irrigate 5–10 hectares per day, and supply a few small village industries.

Although the per KW cost of generating electricity in this way is expensive, Dr Usmani pointed out that there is really no other option. He thought that the present cost would fall because the cost of solar cells was bound to fall further. A decade ago, photo-voltaic cells cost about \$150/W: now, they cost \$15/W even when the total generated power does not exceed 10MW. If demand rises to 500MW the cost may come down to \$1/W, he said. The UN is helping to increase this demand by establishing RECs.

In any case, Dr Usmani said, it was not economically feasible to extend the grid to cover scattered far flung villages in countries like Pakistan. Neither, with the high cost of oil and coal, was it feasible to build oil or coal fired rural power stations for a cluster of remote villages.

Dr Usmani said that his economics for RECs "bear comparison even now, in terms of the cost of power per unit, with that produced by diesel power generators if the price of oil is 20 cents/litre or more and funding of rural electrification is available at a 2–5% rate of interest".

Azim Kidwai

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Camera Press

Village life: light on the way?

European energy demand slows up

ENERGY demand in the European Community increased by 1.1% in 1977 compared to 6% in 1976, according to a report on energy demand in 1977 and the outlook for 1978 by the European Commission for the Council of Ministers. The low level of increase in 1977 is put down partly to the low rate of economic growth, 2% of gross national product (GNP) compared to 4% of GNP in 1976, and to energy savings.

The demand for oil fell by 2% in 1977 to 530 million tonnes. The report

puts most of this down to reduced consumption of fuel oil in power stations because of the increase in nuclear and hydroelectric power. Coal consumption dropped by about 3% to 250 million tonnes of oil equivalent, mainly because of depression in the steel industry and a reduction in the consumption of coke and coal. But more coal was used in power stations.

In contrast to this downward trend, natural gas consumption increased by 6%. Its total share of energy supply rose to 17.5%. Electricity demand

increased by 3.4%, most of the increase coming from the domestic users.

By the end of 1977, total nuclear capacity was 22,400 MW, an increase of 3,300 MW on the previous year. In 1978 nuclear capacity should reach 27,000 MW and contribute 12% of total energy compared to 10% in 1977.

The report forecasts a 3% increase in energy demand in 1978. Oil demand is expected to increase by 2%, natural gas by 9.9% and electricity by 3.9%. Coal consumption should increase marginally. The GNP is expected to rise by about 3%. The Community's annual, medium term target is 4.5–5%.

Counting the costs of US research

PRESIDENT Carter's science adviser, Dr Frank Press, has intervened in a heated dispute between universities and the administration over how much universities should be reimbursed for carrying out federally-sponsored research.

Several years of frequently acrimonious debate, in particular over the allocation of 'indirect' costs such as libraries, utilities and administration, have been brought to a head by last month's publication by the Office of Management and Budget (OMB) of proposed changes in 'cost principles'. These are the rules by which the administration lays down what a university can and cannot charge to a federal agency for carrying out sponsored research projects.

According to OMB, the purpose of the proposed changes is primarily to simplify accounting procedures by providing a common framework for estimating allowable costs that can be applied by all federal agencies who sponsor such research. At present each agency has slightly different rules, making it difficult to prepare an overall picture for public scrutiny.

Furthermore it is claimed that the proposed changes could result in universities having to find up to \$200 million from other sources to sustain research efforts at the current level, a move whose net result might include a reduction in the amount of research carried out.

Recent years have seen a dramatic growth in indirect costs both in real terms and as a proportion of direct costs. Many large institutions now adding 60 to 70% on top of a scientist's grant application. And this has led to criticism from Congress (and frequently from scientists as well) that such costs are not always justifiable.

The changes proposed by OMB are the result of a process begun two years ago when the department of Health, Education and Welfare (HEW), the agency responsible for over half of the \$3.3 billion of federal research and development carried out by US universities, was directed to develop a system for sharing costs 'fairly and equitably' between the two parties.

The response of many universities to OMB's resultant proposals is that, as they now stand, they are neither fair nor equitable, but would impose a greater financial burden than is borne by private corporations of other non-profit institutions, and will, in the words of the dean of research at Stanford University, "further discourage the development of promising young researchers".

Two proposals in particular have been attacked for their financial implications: the first limiting the recovery of library costs to those incurred only by full-time research workers and the second disallowing the costs of providing services for graduate students.

OMB officials say that the new proposals have not been prepared with any conscious intention of placing additional financial burdens on the universities, although admitting that some may suffer by having to adopt less-favourable accounting procedures.

However the debate is not merely financial, but reflects deeper concerns about the politics of basic science. And OMB faces a practical dilemma in its attempts to develop an accounting system that meets both Congress's demands for public accountability and university scientists' claims for flexi-

nor the universities are particularly happy about this, the former claiming insufficient checks and balances, the latter complaining of the burden on research scientists of having to certify the daily activities of laboratory technicians.

OMB's problems reflect a central issue facing the Carter Administration, namely how to meet both Congress's demands for greater public accountability and promises to reduce the amount of government regulations and red tape. The dilemma is particularly acute for a president who has claimed a special awareness of the problems facing the university research community, and has promised to make good the neglect of his immediate predecessors.

"What is so obviously lacking in these proposals in a national policy for basic research in higher education" claim two policy analysts, Frank Riddle and Janet D. Sweet at Stanford University. And it is partly in an effort to refute the charges that the administration is planning to take away with one hand what it is offering with the other, that Dr Press's Office of Science and Technology Policy has now become involved in the debate, attempting to provide a neutral channel of communication between the two sides.

The universities themselves are remaining far from inactive. The 107 member institutions of the National Association of College and University Business Officers (NACUBO) are, with the active support of the Association of American Universities, each preparing detailed estimates of the likely impact of the OMB proposals. The results of these surveys are being discussed this week at hastily-arranged meetings in Washington and Denver, and the conclusions will be presented to OMB at a meeting on 4 May.

Looking towards a longer perspective, the National Science Foundation is busy discussing with universities alternative patterns of research funding, for example investigating whether it would be possible to develop a legitimate way of "pooling" research funds from different sources to provide a particular research group with greater financial stability.

But given the intensity of the present debate, and OMB's insistence that it is not committed to the published proposals and is "quite prepared to change any part of them", its provisional goal of publishing a final version by mid-summer seems increasingly remote.

David Dickson



'Poor Chuck, lost his discovery among the grant application forms!'

bility, a dilemma illustrated by reactions to its proposals for assessing how an individual scientist on a government contract spends his time.

Initially HEW, conscious that Congress is pointing an accusing finger at auditing weaknesses, had proposed a regular system of 'personnel activity reports' based on after-the-fact reporting. University administrators, with the protests of scientists ringing in their ears, proposed an alternative system of pre-established 'monitored workloads' only major changes in which would have to be notified.

OMB has proposed a compromise, with monthly or even weekly activity reports for non-professional staff and student employees, while professional staff can select the monitored workload system. However neither HEW



Pollution in the Persian Gulf

OIL-RICH states surrounding the Persian Gulf are discovering the gremlin of economic growth: pollution. Later this month (24 April) representatives from Bahrain, Iran, Iraq, Kuwait, Oman, Qatar, Saudi Arabia, and the United Arab Emirates meet in Kuwait, under the aegis of the United Nations Environment Programme (UNEP), to see what they can do to clean up the Gulf and its coastline.

The size of the problem is indicated in a document released by *Earthscan* (an information unit supported by UNEP). In 1976, it says, the Gulf had 142 major coastal plants in existence or planned. These included 60 refineries and similar plants associated with oil; 11 cement plants; 8 fertiliser plants; 26 desalination and power plants.

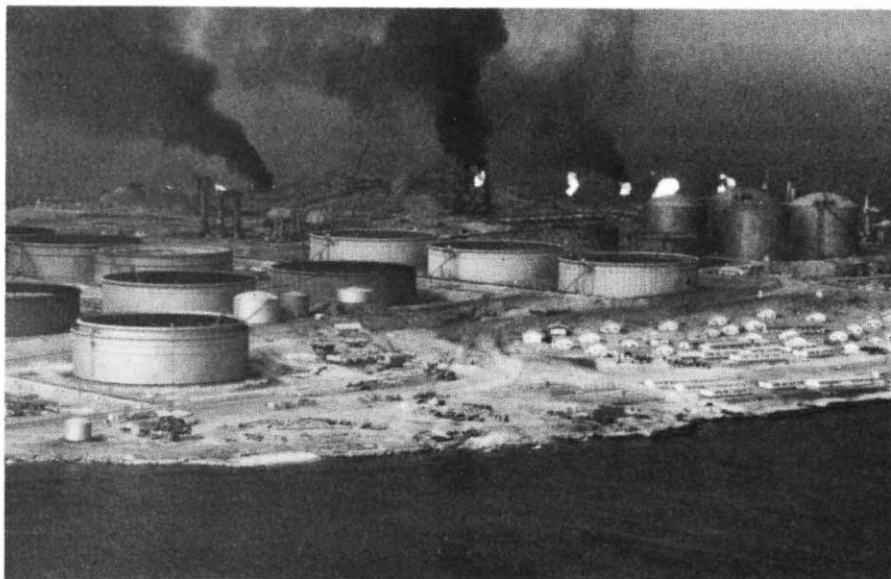
There were also steel mills, aluminium smelters, copper refineries, plastics plants, and a titanium mill, all adding to the pollution load on a sea which averages only 34 m deep, with shore waters less than 10 m deep stretching for many kilometres off-shore.

UNEP concludes: "In this time of development boom, a number of disjointed *ad hoc* projects are under way, often competing for land, materials, and labour, and seemingly of no account in tomorrow's environmental conflicts."

Yet UNEP does not want to impose on the Gulf states environmental controls which will hinder their development. Dr Mostafa Tolba, the Egyptian microbiologist who directs UNEP, told *Nature* last week that he saw no conflict between environment and development. The issue was one of proper environmental management.

It is much more economic, Tolba said, to correct for the side effects of a development plan before it is implemented than to wait until it is too late. "It will be much more expensive and will cause economic de-development, not development."

A specific example would be a cement plant producing, say, 100,000 tonnes of cement a year. This emits 10–20 tonnes of waste materials into the air each day, waste which, according to a UN report, is intolerable to



vegetable crops within several kilometres. The net benefits of the cement plant have to be weighed against its possible costs.

According to the *Earthscan* report the sheer speed of development on the Gulf is part of the problem. Urban populations are increasing at between 6 and 10% per annum, implying a doubling in four to 10 years. (The total population increase is 500,000 a year.)

Spending is high. Industrial investment on the Arabian side of the Gulf averages \$40m per kilometre of coast and \$20m per kilometre on the Iranian side. In Saudi, investment is up to \$100m per coastline kilometre. Moreover, the inevitable future decline of oil revenues in the area is leading to a rapid diversification of industry.

Industries and agriculture need water, and the Gulf is the source and the sink for most of it. It is warm, shallow, long and thin (1,200 km by some 75 to 350 km) and closed by the narrow 62-km Strait of Hormuz. The water balance is maintained from the Gulf of Oman, as the evaporation from the Gulf exceeds the inflow from rainfall and rivers.

Evaporation of the surface waters also causes them to sink, creating pollution traps; and the sea has the world's greatest concentration of desalination plants, sending fresh water to the land but highly saline water to the sea. It is thus not surprising that UNEP believes that the sea's capacity for breaking up and digesting industrial and urban waste is poor.

The coastal ecosystems have been little investigated, but, according to the 1976 UNEP mission quoted by *Earthscan*: "If coastal urbanisation, untreated sewage effluent, industrial wastes and heated water, plus brine discharge from desalination plants continue to increase at the present pace,

many ecological problems will be compounded beyond recovery."

It is against this background that the coastal states meet—and none too soon. According to one Middle East expert they could have come together two years ago, if they had been able to decide what to call the Gulf. The "Persian Gulf" (its usual geographic name) offended the Arabs, the "Arabian Gulf" the Persians, and now even the compromise "Gulf" is rejected by Iran (for its omission of "Persian"). UNEP is now calling the Gulf "The Region".

At the Kuwait meeting, the states will discuss an "action plan" and two environmental protection treaties. The first treaty pledges the states to "prevent, abate and combat pollution" and the second to cooperate in case of emergency. (A shipping accident is not out of the question: the Gulf contained 26 oil-loading terminals in 1976 and 100 vessels a day were entering the Straits of Hormuz. Harbours are so congested that ships have to wait weeks before they unload.)

The centre of the action plan is a programme, similar to a highly successful one in the Mediterranean, to research the extent and effect of pollution in the Gulf. It also deals with development—for example of water management, solar energy, and aquaculture.

Dr Peter Thacher, deputy executive director of UNEP, said recently "The development of a programme for the protection of the sea and coasts [of the Gulf] requires a scientific assessment of the current quality of the environment and a survey of the capabilities of the region's marine scientists and laboratories. The provision of technical assistance, training and equipment will be an essential feature of the programme."

Robert Walgate

Kosmos—1000 up

The Soviet Union has launched its thousandth Kosmos satellite. Vera Rich pinpoints some of the highlights of the series

KOSMOS satellites are not the most publicity-catching part of the Soviet space programme — indeed until Kosmos-954 made an “unscheduled re-entry” into Canada recently, the series had long since faded into the limbo of the unnewsworthy.

Yet, according to one of the creators of the series, Professor V. Mikhailov, speaking on the occasion of the launch of Kosmos-1000, the essentially routine nature of the series represents “a convincing example of the rational approach to the solution of the problems facing cosmonautics”.

The diverse research programme of the series, he said, is based on “several types of unified apparatus”, permitting the “series” production of satellites, irrespective of their particular scientific mission. This virtually mass-produced output of satellites has undoubtedly greatly assisted the high turnover speed of the series, which has reached the 1000 mark in 16 years and two weeks, with more than 500 launches in the past six years.

The Kosmos series began with the launch, on 16 March 1962, of an unnamed satellite, as part of a programme to be carried out “in the course of 1962”, and including:

- study of the concentration of charged particles in the ionosphere with the aim of investigating the properties of radio waves;
- study of the energy states of the radiation belts of the Earth, with the aim of estimating the radiation hazards for long-term space-flights;
- study of cosmic rays and variations of their intensity;
- study of the magnetic field of the Earth;
- study of short-wave radiation of the Sun;
- study of the upper layers of the atmosphere;
- study of the action of meteor matter on space objects;
- study of the distribution and formation of cloud systems in the Earth's atmosphere.

The name Kosmos-1 was applied to this satellite only retrospectively, when three weeks later TASS reported the launch of Kosmos-2. Soon Kosmos satellites were going into orbit so rapidly that the world press grew tired of reporting them—particularly since their purpose was never announced. In the early days, the aim of the programme was repeated from the original TASS statement of 16 March, 1962;

later this was usually reduced to a brief formula. In this respect, Kosmos-1000, which was specifically declared to be a navigational satellite, is something of an anomaly. However, this “new direction in the extensive use of space technology” was doubtless chosen to coincide with the thousand-mark.

The lack of news-interest in the Kosmos series has had a number of advantages from the point of view of the Soviet planners. The name Kosmos is noncommittal: it could be used, if necessary, to conceal a failure or a trial run. Thus Kosmos-41 is thought to have been an unsuccessful communications satellite (or perhaps simply a “dry run” for the later Molniya-1). Kosmos-96 is presumed to have been a Venus probe left (or stranded) in orbit. The mysterious Kosmos-146, which arrived in orbit with a length of approximately 14 metres, and by next day had contracted to about 9 metres after emitting two capsules is thought to have been a Soyuz precursor.

In an interview last week Georgii Narimanov of the Institute of Space Research of the Soviet Academy of Sciences said that Kosmos satellites

have been used to test long-range communications systems, piloted re-entry installations, life-support systems, and assemblies for the lunokhod moon-rover, as well as automatic docking procedures (Kosmos 186–188). Only in the last case, however, could outside observers deduce at the time the purpose of the launch.

The lack of information soon led western observers to speculate about possible military applications — particularly after Kosmos-57, which was launched on 22 February, 1965, and which blew up (or was blown up) the same day, but which was reported by TASS the following day to have “all systems functioning normally”. Such speculation can, of course, lead to false conclusions; a number of events in the Kosmos series are most easily explained as hunter-killer tests, one satellite exploding in the vicinity of another (though without the direct attack which would contravene international agreements banning weapon testing in space). The Soviet Union, of course, denies all allegations that it is developing satellite-killing satellites, attributing this “myth” to opponents of a new SALT agreement who wish to “obstruct progress towards the completion of such an agreement”.

The wide-spread speculations at the end of 1976 that killer satellite tests are aimed at Chinese, rather than US reconnaissance satellites, being carried out at a relatively low altitude, have

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Novosti

Right: a Kosmos satellite Left: lift-off of a Kosmos carrier-pocket in assembly with the intercosmos satellite (1969-73)

proved premature. Subsequent tests of this nature have taken place at a wide range of altitudes, including those favoured by the US satellites. The presence of a nuclear pile, rather than an isotope source, aboard the ill-fated Kosmos-954 led to speculation about high-powered radar installations; since, however, Soviet scientists are currently working on the use of radar for oil-slick detection, Kosmos-954 might well have been engaged in monitoring nothing more sensitive (in the military sense) than environmental pollution.

Certainly the "eight-day" satellites, which re-enter within a few days of launch and before their orbit has naturally decayed suggest some kind of photographic reconnaissance operation—particularly since they typically have fairly low altitudes. Undoubtedly, the general name Kosmos could provide a cover-all for a number of undisclosed purposes—not all military (there has been recent speculation that Kosmos-929 was in fact the test flight of a space-tug, particularly in the light of evidence that the Soviet Union is working on a reusable space-shuttle).

In fairness to TASS and the Soviet authorities, it must be admitted that the majority of Kosmos research is more scientifically valuable than newsworthy. Its place is in journals such as *Kosmicheskie issledovaniya*, not the headlines of *Pravda*.

Such news as is released to the general press is of the more eye-catching type—the laser experiments on Kosmos-97, the biomedical experiments (using dogs) of Kosmos-110, meteorological photographs from Kosmos-122 and -144. The launch of Kosmos-122 was witnessed, incidentally, by General de Gaulle on his visit to the Soviet Union—the only non-Socialist head of State to be so honoured. And the announcement that the originally routine Kosmos-5 (launched 28 May, 1962), had picked up high altitude radiation from the USS "Starfish" nuclear test (9 July, 1962), provided a valuable incentive towards the conclusion of a test-ban treaty.

There is now a serious lag in the publication of scientific results from Kosmos satellites. Typical papers published in *Kosmicheskie issledovaniya* in 1977 present data from Kosmos 225, 261, 348, 391, 426, 461, 484 and 490.

Broadly speaking, the more important it is for future developments, the more likely it is that a given satellite will receive an early "write-up". Thus the cosmic-ray shielding system tested on Kosmos-605 was reported in *Kosmicheskie issledovaniya* No. 5, 1975. This system, to judge by the diagram (a cutaway sketch of a shielded cabin, with a human figure at the centre), is designed for a manned station.

The effects of radiation exposure have been a cause of worry ever since the flight of the dogs "Veterok" and "Ugolek" in Kosmos-110. This satellite was orbited high in the Van Allen belts, possibly to test a new "antiradiation serum". Little is known about biological experiments for the next few years; however, Dr N. N. Gurovskii observed (on the occasion of the launch of Kosmos-1000) that the biomedical work of the Kosmos series had "facilitated such notable achievements" as the flight of the Vostok, Voskhod and Soyuz spaceships and the Salyut spacestation—suggesting an on-going programme throughout the 1960s.

At the time, however, it appeared that there was a lull in the biomedical aspect of Kosmos, until October 1970, when Kosmos-368 carried "scientific apparatus for testing experimental life-support systems for laboratory animals, for the future study of space-flight on living organisms, and for the continuation of the investigation of the physical characteristics of space".

Whatever the routine biomedical work in the meantime, this aspect of the Kosmos series came, once again, to the fore with Kosmos-782 launched at the end of 1975. This was an international event, carrying experiments not only from the Socialist block (Poland, Czechoslovakia, Hungary and Romania), but also France and America, as well as the Soviet contribution. The main concern of Soviet space medicine is the genetic hazard—hence this craft carried *drosophila* (once banned to Soviet research by Lysenko) as well as seeds and unicellular organisms. Higher life forms included tortoises, rats and fertilised fish. A special Soviet-US cooperation experiment investigated the effect of space conditions on tumour cells.

A second such 'international bio-laboratory' was launched as

Kosmos-936 in August, 1977. Special emphasis was placed in this flight on "problems of radiation safety in protracted space flights". A TASS correspondent, Nikolai Zheleznov, commented that the work of US and Soviet cosmonauts on board the Salyut and Skylab orbital stations had now "removed from the agenda" the question of whether man can leave the Earth for a long period. The main medical problem, he said, is now to ensure the proper functioning of a "space dwelling" in all respects from safe "habitation conditions" to the provision of consumables. A special feature of the biolaboratories (though there are conflicting reports as to when it was first introduced), is a gravity-simulating centrifuge allowing the effects of reduced as well as zero gravity to be investigated.

The main purpose of the Kosmos series, remains, however, geophysical and astrophysical surveys. In accordance with current Party doctrine, which stresses the service of science to the community, it is continually stressed that the whole programme aids the national economy by giving a better understanding of natural environmental processes, as well as providing data for geological surveys, weather-forecasts, and the mapping of the movements of fish shoals.

The individual experiments may be apparently abstruse—but officially the whole Kosmos programme (as, indeed, the whole space programme, manned and unmanned) is designed to serve the national economy.

How, then, has the economy benefited? To mark Kosmos-1000, *Pravda* published a kind of symposium of comments by those closely connected with the programme. Academician Obukhov emphasises the practical aspect: "The experience accumulated in the course of the geophysical experiments of the 'Kosmos' satellites was used in the concrete development and realisation of the 'Meteor' meteorological system". Others stress the basic research: "The launch of Kosmos-321 gave a picture of the distribution of the magnetic field over 94% of the surface of the terrestrial globe" (Dr Sh. Dolginov); "For the investigation of the upper atmosphere, the aurora, and the magnetosphere, the 'Kosmos' programme made direct measurements in circumterran space a reality" (Dr Yu. Gal'perin).

Pravda's concluding comment was, however, entirely utilitarian: "The satellites of the Kosmos series have made and are continuing to make an enormous contribution to science and technology, and facilitate the further practical use of circumterran space for the good of mankind". □

... and one down

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The Kosmos that crashed on Canada earlier this year, on show to the press.

correspondence

Shining light on intelligence

SIR.—I agree with Hermann Bondi (16 March, page 204) that “a great deal of heat and little if any light has been generated by discussions of the inheritability or otherwise of intelligence” but I consider that the rest of his letter functions only to cloud the issue, obscuring the little light that exists.

If we attempt to pierce the clouds, it would appear that Bondi's contribution to the discussion takes the following form. (1) complex human traits have a smaller genetic component than simple ones; (2) mental attributes are more complex than physical ones; (3) therefore, from (1) and (2), mental attributes are more affected by the environment and less by one's genes than are physical attributes; (4) the environment has a “commanding” part to play in athletic ability; (5) therefore, from (3) and (4), genes can have little part to play in determining human intelligence.

Statement (1) is a little difficult to discuss as Bondi does not define complexity. However, he says some traits “(for example colour of eyes or hair, colour blindness and haemophilia) are easily defined, readily measured, constant throughout life, and it seems to be reasonable to classify them as simple”. It is difficult to resist the temptation to question whether hair-colour really satisfies the three criteria laid down but let us accept that these characters are, indeed, simple. Since they are largely under genetic control, they fit in with statement (1). However, two other simple characters that come readily to mind, handedness and tongue-rolling, appear to have very little genetic basis. Statement (1) may be true in general but there are so many exceptions that one cannot use it to predict heritability from “complexity” in any particular case.

Statement (2), at least when referring to physical achievements such as athletic ability, is pure assumption. Though among readers of *Nature* it may be true that “few would deny” it, this reflects an understandable prejudice rather than the existence of objective data.

Statement (4) rests on a clear misunderstanding of how the interplay of nature and nurture can be studied. The “inheritability” of quantitative characters may be measured as the proportion which the additive genetic variance represents of the phenotype variance. This measure—heritability—may not always coincide with what the non-geneticist means by “inheritability” but it is useful in genetics which is, after all, the scientific study of inheritance.

Bondi's argument, based on the difference in athletic performance between East and West Germans is that the environment must have a commanding part to play in establishing complex physical achievements. If I take two genetically identical populations of an organism, subject them to different environments, and then find that the

individuals with the highest scores for some quantitative trait generally come from one of the populations, I can reasonably conclude that the imposed environmental differences have resulted in the observed differences between the populations. But I cannot tell what proportion of the total variance of the trait in question is due to the environmental difference: it may well be small. Indeed, the variance within each population may be entirely genetic. To estimate either the overall heritability or the heritability within populations we need quite different data. This is as true of Bondi's particular case as of the general case.

It would thus appear that statements (1), (2), and (4) are almost totally unfounded. Conclusions (3) and (5) must, therefore, fall. They may eventually turn out to be correct but Bondi's arguments do not help us decide one way or the other.

The problem with measuring the heritability of intelligence is not that it is very small or that the question is complex—after all, we can measure the charge of an electron and discover the amino-acid sequences of proteins. The real problem is that, for perfectly proper reasons, we cannot do the usual experiments. For traits with heritability close to unity, this matters little. For all others, not just intelligence, it matters a lot. Is Bondi advocating that because this problem is consequently difficult, we should not try to solve it?

J. J. D. GREENWOOD
University of Dundee, UK

SIR.—The argument advanced by Sir Hermann Bondi (16 March, page 204) leading to the conclusion that environment “must have a commanding part in establishing complex human physical achievement” is unsound. In the first place comparisons of small numbers of exceptional individuals (such as Olympic athletes) are not particularly informative with respect to the qualities of the population from which they are drawn. More importantly, however, while the athletic achievements of the DDR and some other nations of Eastern Europe are indeed outstanding it seems likely that this owes not a little to the highly selective (not to say elitist) practices which operate in many of these countries. In such an environment individuals displaying creative, intellectual or physical talent tend to be systematically picked out and coached from an early age.

It could thus be argued that the DDR is better than, say, the Federal Republic of Germany at recognising outstanding genotypes and that environmental contribution to the achievements of such individuals is therefore rather small.

Both Sir Hermann's conclusion and the one derived above are overly simplistic. There are other, and scientifically more respectable, viewpoints which take account of the importance of the right environment for the right genotype in

achieving an extreme value for any aspect of phenotype. The truth can, of course, only be established by properly planned and executed enquiry from which we should not be deterred by the “incredibly complex” nature of the physical and mental attributes involved.

Yours faithfully,
J. R. BEARDMORE
University College of Swansea, UK

Windscale: preparing MPs for an informed debate

SIR.—Your editorial (30 March, page 389), on the Windscale debate makes a perfectly fair point that an issue of this kind cannot be satisfactorily disposed of by a few hours slanging match across the floor of the House. The subject highlights the need for a powerful and coherent committee system for the House of Commons which at present it lacks.

However, your comments are a good deal less than fair to the work of the Select Committee on Science and Technology, of which I am a member, and which has produced over the past few years a number of important and influential reports on energy problems including a recent one on alternative energy sources commending the development of tidal, wave and solar power. I am inclined to agree that energy is now a subject of sufficient importance to deserve a Select Committee in its own right.

Your editorial does dodge the issue as to who must take the ultimate decision in a matter as important as Windscale. A Select Committee can probe, investigate, weigh evidence and make recommendations, but the House itself must ultimately decide. In my view, the vote before Easter of 186 to 56 probably represented fairly well the current balance of opinion within the House of Commons on the nuclear issue—which does not split on party lines. Nevertheless, I would hope that more work of the kind already carried out by the Select Committee on Science and Technology taken together with the work of other bodies such as the Royal Commission on Environmental Pollution, will eventually bring about a substantial shift in this balance.

Yours faithfully,
FRANK HOULEY, M.P.
*House of Commons,
London, UK*

Argentinian haematologist released

News has just been received that the haematologist Dr Beatriz Iparaguirre-Weinstein, who was reported missing in Buenos Aires (30 March, p 396) has now been released by her captors and is safe.

news and views

10 nm filaments

from David Gilbert

COMPARED with the thick and thin filaments of myosin and actin found inside cells, 10 nm filaments (also known as intermediate, 100 Å or decofilaments, or, in various tissues, as neurofilaments, glial filaments and tonofilaments) languished in obscurity, because they are largely insoluble and lack specific structural and chemical markers. Now this is rapidly changing, thanks to new preparative procedures and to fluorescent antisera.

Early 10 nm filament preparations came first from squid giant axons (with difficulty, see Huneus & Davison, *J. molec. Biol.* **52**, 415; 1970), then from vertebrate brain (Shelanski *et al. Science* **174**, 1242; 1971). Neither preparation is yet fully understood. The squid 65 K filament chain seen on SDS gels is now thought to be accompanied by a 200 K chain (Lasek & Hoffman, in *Cell Motility*, Cold Spring Harbor Laboratory, 1021; 1976; Gilbert *J. Physiol.* **266**, 81P, 1976); whereas the brain preparation contains a mixture of immunologically distinct neurofilament chains, both of molecular weight ~50 K (Eng *et al. Proc. Int. Soc. Neurochem.* **6**, 100; 1977), in addition to larger neurofilament chains (Lasek & Hoffman *op. cit.*). Another giant axon, from *Myxicola*, gives over 20 neurofilament chains, all apparently derived from *in vivo* proteolysis of a 160 K 'intact' chain. Similarly, Steinert *et al. (J. molec. Biol.* **108**, 547; 1976) showed that keratin tonofilaments can be reassembled from a variety of native chains of molecular weight 47–58 K.

Two recent preparations, thankfully of simpler compositions, are based on the insolubility of vertebrate 10 nm filaments. Cooke first exploited this property to extract the 10 nm filaments from smooth muscle. (*J. Cell Biol.* **68**, 539; 1976). This 55 K protein was further characterised biochemically by Small and Sobieszek (*J. Cell Sci.* **23**, 243; 1977) and named 'skeleton', after its evident cytoskeletal function, then analysed immunologically by Lazarides and Hubbard (*Proc. natn. Acad. Sci. U.S.A.* **73**, 4344; 1976), and named 'desmin' (the papers crossed in press), after its possible role in the mechanical linkages between muscle cells. Its chain weight also varies slightly from species to species (Lazarides & Balzer *J. Cell*

Biol. **75**, 255a; 1977).

In the second new preparation colchicine is used to induce in cultured cells a thick, 'juxtanuclear cap' of 10 nm filaments. These pellet conveniently after cell lysis and yield two chains (54 K and 55 K) migrating close to α -tubulin, although they do not cross react immunologically with tubulin (Starger & Goldman *Proc. natn. Acad. Sci. U.S.A.* **74**, 2422; 1977). Parenthetically, this migration in SDS gels of filament protein just in front of, on top of, or behind tubulin (depending on the author) has spawned many controversies, because it makes contamination by tubulin, or by other bands in this crowded region of the gel, difficult to detect.

Are all these 10 nm filaments related? Biochemical studies have failed so far to pinpoint real similarities. The variations in chain weight could be due to proteolysis, since, for example, filaments from both giant axons are rapidly cleaved into ~50 K fragments by native proteases (Anderton *et al. Biochem. Soc. Trans.* **4**, 544; 1976). This hypothesis now seems unlikely to account for all the observed variations, because peptide maps (Davison *et al. Expl. Cell Res.* **109**, 471; 1977) have yet to show any compelling similarities between brain and skeleton chains. Nevertheless, the results discussed below suggest that further work in this area will be well-rewarded.

In contrast, immunological studies, which are insensitive to the vagaries of chain weight that dominate the biochemical picture, suggest profound and widespread similarities within the ~55 K class of 10 nm filaments. To judge from recent output, this exciting prospect has kept many ultraviolet arcs burning far into the night.

Just as control over the ~55 K antigens is fairly tenuous, for the reasons given above, so antisera raised to them are doubly suspect. No one has yet raised antisera fulfilling the criteria of, say, Fugiwara and Pollard (*J. Cell Biol.* **71**, 848; 1976) even though large

fractions of the following papers are devoted to establishing links between 10 nm filaments, the antigens and the antisera. All authors make plausible cases corroborated by electron microscopy, but perhaps more compelling is the fact that diverse antisera in several laboratories give immunofluorescent patterns which consistently parallel the distribution of 10 nm filaments.

Fluorescent networks of 10 nm filaments were first shown by Jorgensen *et al. (Proc. natn. Acad. Sci. U.S.A.* **73**, 3192; 1976) in one of the better-controlled studies. They raised antisera to the complex 55 K brain band described above, and showed wavy bundles clustering near the nucleus of neuroblastoma cells and extending into the longer cell processes. The bundles did not stain with antisera to tubulin or to tropomyosin. Lazarides' and Hubbard's (*op. cit.*) antisera to skeleton showed intense fluorescence near Z-lines and elsewhere, suggesting that this protein helps transmit tensile stresses between muscle fibres.

Coiling of 10 nm filament bundles into the 'juxtanuclear cap' after treatment with demecolcine was next introduced by Blose *et al. (Proc. natn. Acad. Sci. U.S.A.* **74**, 662; 1977) as an additional assay, subsequently used by most groups, for 10 nm filament specificity. They also used the 55 K brain filament band as the antigen, and successfully stained both endothelial and cardiac muscle cells. 10 nm filaments therefore appeared to be both highly conserved and widely distributed, an important hypothesis borne out by the more extensive cross reactions to come.

Antibodies to 10 nm filaments also arise spontaneously, and these sera present much the same fluorescence patterns: Osborne *et al. (Proc. natn. Acad. Sci. U.S.A.* **74**, 2490; 1977) used one such serum to contrast the wavy bundles of 10 nm filaments, and the roughly similar pattern of microtubules, with the straight 'stress fibres' of actin. Cytochalasin disrupts the latter selectively, while demecolcine disrupts only the microtubule pattern. Human and rabbit autoimmune sera (Kurki *et al. Nature* **268**, 240; 1977; Gordon *et al. Cell* **13**, 249; 1978) also stain 10 nm filaments in a wide variety of cells, according to these same criteria. Gordon *et al.* identify a 56 K protein as the primary antigen, using a novel gel technique, while Kurki *et al.* can absorb out all activity from their serum with

skeleton. Therefore some protein(s) of ~55 K are clearly implicated in 10 nm filaments from a wide variety of cells.

10 nm filaments, unlike those of actin and tubulin, seem not to be closely involved in locomotion, cell spreading or mitosis; rather they lag behind microtubules and actin when the cell moves, and coil in the periphery (though not in the intercellular bridge) when the cell divides. They are apparently polymerised at all times. These insights come from the work of Hynes and Destree on cultured fibroblasts (*Cell* **13**, 151; 1978) using a major 58 K cytoskeletal protein from the fibroblasts as the antigen. Thus fluorescent labelling is beginning to limit the hitherto speculative functions of 10 nm filaments.

Complementing and confirming these immunofluorescent studies are two recent reports in which resolution is extended to electron microscopic levels by detergent extraction of cultured cells, a technique elegantly developed by Brown, Levinson and Spudich (*J. supramolec. Structure* **5**, 119; 1976). Both support a contention made by Metuzals and Mushynski (*J. Cell Biol.* **61**, 701; 1974) that 10 nm

filaments connect importantly with cell nuclei. Lehto *et al.* (*Nature* **272**, 175; 1978) show that, at the very least, nuclei are mechanically trapped tightly inside a cytoskeletal cage of 10 nm filaments. Small and Celis (*J. Cell Sci* **31**, 393; 1978) reach a similar conclusion on the basis of negatively-stained whole cells, from which sufficient membranes and soluble proteins were extracted to allow direct visualisation of remarkable 'Triton cytoskeletons' composed in large part of 10 nm filaments.

Interestingly, both groups (with Lazarides and Balzer, and Starger and Goldman *op. cit.*) find small amounts of actin remaining in these cytoskeletons, despite extraction with buffers. This suggests a tantalising link between 10 nm and thin filaments.

Significant questions remain: first, are all the ~55 K antigen proteins related to each other, to keratin, or to the curiously high molecular weight neurofilaments? Phrased another way, how many varieties of 10 nm filament are there? In the longer term lie the challenges of reassembly, enzymatic modification, copurifying minor proteins, and most elusive of all, function.

Tree-ring chronology

Peter D. Moore

PALAEOCLIMATOLOGISTS, dendrochronologists, archaeologists, indeed all European Quaternary environmental historians must currently be looking hopefully towards the Palaeoecology Laboratory of Queen's University, Belfast, for the answers to a number of important problems. Pilcher, Hillam, Baillie and Pearson, of that laboratory have now published long-awaited details of their floating tree-ring chronology from the sub-fossil bog oaks of Northern Ireland (*New Phytol.* **79**, 713; 1977).

The work in Belfast began about 10 years ago when the discovery of large numbers of oak tree remains, well preserved in anaerobic peat deposits, opened up the possibility of developing a tree-ring chronology similar to that constructed for bristlecone pines preserved under arid conditions in the White Mountains of California. Any site with a large number of tree trunks preserved is likely to span several centuries, and their technique has been to develop a cross-correlation of rings between individual trees at a site and hence to construct a local, floating site chronology. Radiocarbon dating of woods between sites has assisted in matching these floating chronologies into one master chronology.

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Initially, cross-matches were made visually, but were confirmed by computation of a correlation coefficient at each possible ring match. The maximum correlation was given by the best match. Within any site, depending on the length of time over which trees had grown and had been preserved there, it was often found that there was no overlap between certain individuals. Only when a number (perhaps 10 or 20) of trees had been sampled could a complete site chronology be built up. One site, Garry Bog, had trunks which in total spanned 1,500 years (56 trees) and another, the Lough Neagh fenslands, covered nearly 1,700 years (134 trees). It is interesting to note that the Lough Neagh trees had a much shorter life than those at Garry Bog, with a mean life span of 180 years as opposed to 220 years at Garry Bog. Presumably these data are biased towards the larger trunks surviving in the peat; a more random sample could provide some interesting information concerning the demography of oak on these different sites.

In developing a master chronology for Northern Ireland, the curves are effectively filtered of their long-term trends by fitting a polynomial and reducing the yearly data to indices of departure from the polynomial. Medium term trends (20–40 years) are preserved and the site chronologies are

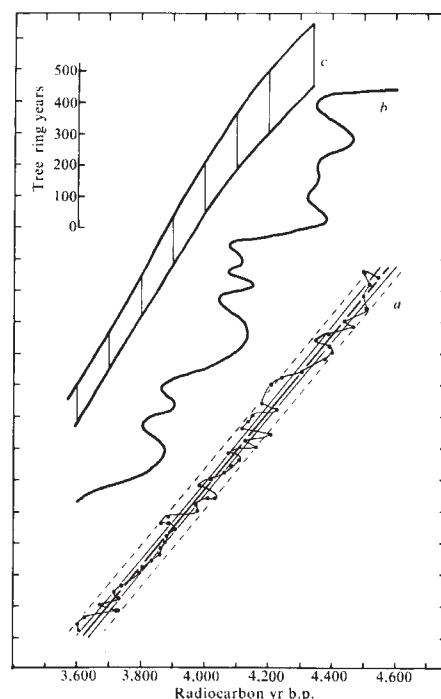


Fig. 1 Relationship between radiocarbon and tree-ring years. *a*, Relationship established using low-altitude oak from the North of Ireland; two standard deviation limits dashed, regression line drawn with curvilinear 95% confidence limits. *b*, Curve published by Suess (*Radiocarbon Variations and Absolute Chronology* (ed. Olsson, I. U.) 303, Wiley, New York, 1970) based on bristlecone pine wood. *c*, Historically derived calibration based on Egyptian material published by McKerrell (*Radiocarbon: Calibration and Prehistory* (ed. Watkins, T.) 47, Edinburgh, University Press, 1975).

found to match well.

The master chronology is 'floating' in the sense that it cannot be linked with the present day by cross-correlation with further tree-ring data. Radiocarbon dating of samples suggests that it extends from about 2,950 b.p. back 2,990 years to about 5,950 b.p. Further data are now available going back beyond 7,950 b.p., but cross-correlation of the additional site data has yet to be ratified. Forwards in time, there remain three gaps which must be bridged before the floating chronology can be firmly anchored to the present day. The gap at about 2,950 b.p. is of particular interest and concern in that it seems to represent a time when the persistence of many upland sites terminated abruptly. This may have been caused by climatic changes or by a change in land use on the part of Bronze Age man. It is interesting to note that Smith (*Council for British Archaeology Report No. 6*; 1976) finds that many of the blanket peats of Northern Ireland began to form at about this time.

The radiocarbon dating studies of

these Irish bog oaks have themselves provided some interesting and controversial data. In a recent publication from the Belfast Palaeoecology Laboratory, Pearson *et al.* (*Nature*, **270**, 25; 1977) describe in detail the methods they have used for dating contiguous 20 year increments taken from different trees. They have modified their technique to minimise the many sources of error in conventional radiocarbon dating. They use a scintillation counting technique, having converted their generated CO_2 to benzene, and are able to quote a final standard deviation of 25 years. This high degree of precision compares favourably with the most accurate gas counting systems.

But the most important feature of their plot of radiocarbon years against the floating tree-ring scale is the low amplitude of fluctuations exhibited around a linear regression line which is drawn through the data. As they point out, the familiar Suess calibration 'wiggles' are far less prominent in their data than in the published Arizona material, and their curve resembles more closely the calibration based upon Egyptian material. On the basis of this observation, they feel that more accurate dating will lead to a closer approach to a linear calibration for the radiocarbon chronology.

Suess (see *Matters Arising* in this issue, page 649) challenges this conclusion. He feels that the fluctuations which remain in the Belfast data are

disguised by the fitting of a linear regression and that these wiggles can be matched with the Arizona data. His calibration curve, Suess claims, fits the observed data from Northern Ireland better than the proposed straight line. The fact that all determinations fall within the 95% confidence intervals of a linear model, however, indicates that the present level of precision, impressive though it may be, is statistically inadequate for the detection of any real wiggles which may exist. If Suess's claim that the Northern Ireland wiggles match those described for Arizona can be substantiated, however, then this would give strong support to the belief that the wiggles are real. As Pearson *et al.* reply, we need to see this demonstrated. We must also wait for the publication of other correlations between American and European work to which Suess alludes. The amplitude of oscillation appears lower, but it is still possible that European calibration curves exhibit the Suess wiggles.

Meanwhile, the value of European oak tree ring widths as indices of former climatic conditions is supported by the analyses of Hughes *et al.* (see page 605 in this issue of *Nature*). In two out of three sites examined they find that over 60% of the variance between tree-ring widths can be accounted for by climatic variables. This provides confidence for the use of such data in detailed palaeoclimatic reconstruction. □

Gazzetta Sanitaria **23**, 11; 1974). Indeed, in areas of the world where the prevalence of macronodular cirrhosis and primary liver cancer is high, infection with hepatitis B virus and the carrier state occur most frequently in infants and children, and as many as 20% of the general population may be carriers. It appears likely, therefore, that persistent hepatitis B virus infection occurs before the onset of chronic liver damage.

Another possibility is that persistent infection with hepatitis B virus leads to cirrhosis and that carcinoma then arises from regenerative nodules by mechanisms in which the virus is not involved. Such a mechanism may account for cases of liver cancer which occur in patients with alcoholic cirrhosis. However, this sequence does not explain liver cancer associated with persistent hepatitis B infection in about 25% of patients in the absence of cirrhosis.

There is now additional evidence of a close association between hepatitis B and liver cancer. For example, Macnab and her associates (*Br. J. Cancer* **34**, 509; 1976) derived a cell line producing hepatitis B surface antigen from a South African patient with primary liver cancer. Supernatant fluids from these cultures contain the surface antigen only and the antigen titre increases with time. A variety of sensitive techniques have not yet detected complete virus particles or indeed the cores or nucleocapsid of the virus in the cells nor in the supernatant fluid. This implies the possible integration of part of the viral genome into the host cell genome resulting in production of excess viral coat protein.

A radioactive copy of ~25–50% of the hepatitis genome can be made using the endogenous viral DNA polymerase and can be used as a probe for viral sequences in the DNA extracted from the livers of patients with both hepatitis B infection and hepatocellular carcinoma (Lutwick & Robinson *J. Virol.* **21**, 96; 1977). Compared with DNA from uninfected liver, DNA and RNA from these patients hybridised more strongly with labelled hepatitis virus DNA indicating the presence of viral sequences. The finding of virus sequences in the rapidly sedimenting DNA from the infected liver suggests that some of the sequences are probably attached or integrated into the host DNA. Other experiments indicated that viral sequences can be detected in RNA extracted from liver infected with hepatitis B virus but not in RNA from uninfected liver. These findings are consistent with transcription of viral DNA in infected liver cells.

The high rate of infection with hepatitis B virus in certain defined popula-

Oncogenic potential of new viral vaccines

from Arie J. Zuckerman

THE safety of any viral vaccine especially in relation to its possible oncogenicity must be carefully considered. The main source of danger may be possible contaminants of the tissue cultures used for the preparation of such vaccines, but in addition a number of viruses, particularly members of the herpesvirus group such as herpes hominis type 2 and EB virus, have been implicated in the aetiology of certain human cancers. Such difficulties have been met, for example, in the development by Elek and Stern (*Lancet* **i**, 1; 1974) of a vaccine against mental retardation caused by cytomegalovirus infection *in utero*, and the problem was summarised by McDougal and Harnden (*Lancet* **i**, 135; 1974). Cytomegalovirus is a member of the herpesvirus group. This matter has also been raised at a recent symposium on hepatitis held in San Francisco in

relation to the experimental hepatitis B vaccines now under development. Hepatitis B virus is an unclassified DNA virus.

The geographical pathology of primary cancer of the liver presents intriguing epidemiological and demographic features. Numerous studies in many parts of the world, particularly in Africa, Asia, the Pacific and certain Mediterranean areas, show a highly significant excess of hepatitis B surface antigen and core antibody in patients with primary liver cancer. These results may be interpreted in several ways. Hepatitis B is ubiquitous in areas where macronodular cirrhosis and primary liver cancer is common, and it is possible that patients with hepatocellular carcinoma are unduly susceptible to infection with hepatitis B and to the development of the persistent carrier state. However, it has been suggested that the important factor in the possible causal association between hepatitis B infection and liver cell cancer may lie in an early age exposure to infection (Zuckerman & Dunne

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tion groups in the developed countries and among the general population in many developing countries indicates the urgent need for a hepatitis B vaccine. The repeated failure to grow and passage hepatitis B virus serially in tissue culture has however hampered progress towards the development of a conventional vaccine. Since the separated surface antigen of hepatitis B virus leads to the production of protective antibody, the possibility of using this material seems feasible. Such experimental vaccines have now been prepared from the plasma of apparently healthy carriers of hepatitis B virus (*News and Views* 255, 104; 1975; 267, 578; 1977). Susceptible chimpanzees

have so far been shown to be protected by such preparations treated with formalin and initial tests in volunteers of these experimental subunit vaccines are now in progress.

However, before large scale clinical trials of the new unconventional hepatitis B vaccines are undertaken or serious consideration given to the suggestion of using these vaccines in children in the developing countries, it is essential to ensure that any benefit that accrues from immunisation is not outweighed by a cancer hazard. Fortunately the necessary sophisticated technology to ensure that no live virus is present in vaccines prepared from serum of carriers is now available. □

Ribosomal genes dissected

from B. E. H. Maden

THE ribosomal RNA genes that occur in the *Xenopus* genome as some hundreds of tandem repeats are amongst the most intensively studied eukaryotic genes at the molecular level. The rDNA repeats consist of alternating transcribed regions and non-transcribed spacer regions (see figure). The transcribed regions consist of the coding sequences for the 18S, 5.8S and 28S rRNAs separated by transcribed regions that are degraded during ribosome maturation. Recent work has elucidated the structure of the non-transcribed region, identified the site of initiation of transcription, and provided information on the methylation pattern of the rDNA.

Evidence from cleavage with the *Eco*RI restriction enzyme has shown that the non-transcribed spacer differs in length from repeat to repeat, whereas the transcribed region remains constant. The molecular basis of this length heterogeneity has now been analysed by Wellauer, Dawid and their colleagues (Wellauer *et al. J. molec. Biol.* 105, 461; 1976; Botchan *et al. Cell* 11, 599; 1977).

Most of the non-transcribed spacer consists of two related domains, B and D, each of which consists of numerous imperfect repeats of a basic sequence

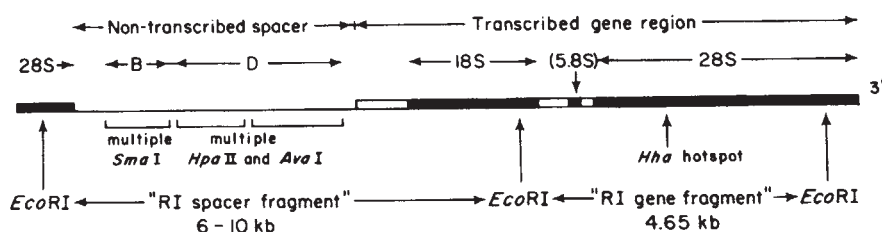
some 15 nucleotides long. Although B and D have a limited sequence homology they can easily be distinguished by restriction analysis. For example region B possesses a very high concentration of cleavage sites for the endonuclease *Sma*I (which recognises the sequence CCCGGG). Region D contains many fewer *Sma*I sites, arranged in a different pattern. This simple sequence DNA resembles in its general properties some other non-transcribed DNAs such as the spacers between genes coding for 5S RNA in *X. laevis* and the centromeric satellite DNA. The overall length heterogeneity of the non-transcribed spacer has been traced to different amounts of these repetitive sequences—D being more variable than B.

Transcription proceeds in a 18S→28S direction and Reeder *et al.* have now located the site of initiation of transcription by the ingenious method of labelling the primary 40S transcription product *in vitro* at the 5' end using the vaccinia virus 'capping' enzyme complex. This adds 7-methyl guanosine in triphosphate linkage to the 5' end of the molecule, and is specific for RNA molecules bearing a 5' terminal di or triphosphate. The capped sequence was found to hybridise to a DNA site some

800 nucleotides 'upstream' from the 18S gene and close to the D region of the non-transcribed spacer, as determined using a number of restriction enzymes. The simple repetitive sequence of the D region ends at least 200 nucleotides before the site of transcription initiation (Reeder *et al. Proc. natn. Acad. Sci. U.S.A.* 74, 5402; 1977; and 10th Miami Winter Symposia, 1978).

Information on quite a different subject has recently emerged from restriction analysis of uncloned rDNA from frogs (Bird & Southern *J. molec. Biol.* 118, 27; Bird *J. molec. Biol.*, 118, 49; 1978). rDNA from frog somatic cells contains some 13% of its cytosine as 5-methyl cytosine (m⁵C), whereas amplified oocyte rDNA is unmethylated (Dawid *et al. J. molec. Biol.* 51, 341; 1970). Cloned rDNA is also unmethylated. Available evidence indicates that m⁵C is the only methylated base in eukaryotic DNA, and that in vertebrates m⁵C is largely confined to the doublet CpG. The doublet CpG occurs relatively infrequently in bulk vertebrate DNA, but with much higher frequency in rDNA (Russell *et al. J. molec. Biol.* 108, 1; 1976). It therefore seemed likely that the high content of m⁵C in somatic rDNA might be due to methylation at the large number of CpG doublets. This possibility was examined by Bird and Southern.

Four restriction nucleases with specificities for CpG-containing sequences were used: *Hpa*II (CCGG), *Hha*I (GCGC), and *Ava*I and *Hae*II, which possess slightly more complicated specificities. The results were striking. Amplified or cloned rDNA was digested into a large number of small fragments, which for the first three enzymes represented some 270 cleavage sites, or just over one quarter of the total number of CpG sites for the average repeat length. In somatic rDNA from erythrocytes almost all these sites were resistant to digestion. Since restriction nucleases are inhibited by DNA methylation, the results indicated that the resistant sites were methylated, to the extent of 99% per site. There were two exceptions to the general resistance to cleavage. One sharply defined site in the 28S coding region was cleaved by *Hha*I in some 50% of somatic DNA repeats, and was termed the 'Hha hot spot'. This site is therefore selectively deficient in methylation. The other region is located in the non-transcribed spacer and was termed the 'Hpa Ava hot spot'. Analysis of this region in cloned DNA revealed an extremely high concentration of cleavage sites for these enzymes. From its location the region evidently coincides with region D of Botchan *et al.* It is not clear whether cleavage of



somatic rDNA in this region is due to selective submethylation at one or two sites, or to marginally incomplete (99%) methylation at each of a large number of sites.

The doublet CpG is self-complementary. The question arises whether m³C occurs on one strand or both. Bird therefore carried out strand separation upon somatic rDNA and reannealing to excess cloned rDNA. If both somatic strands were methylated, both strands should protect the resulting heteroduplexes from digestion by restriction enzymes. This was found to be the case. The finding suggests that m³C formation occurs on the newly synthesised DNA strand during or soon after replication. This was confirmed in *Xenopus* cultured kidney cells by methyl labelling, combined with DNA density labelling to distinguish between parent

and progeny strands: methyl label was incorporated exclusively into the progeny strand. Taken together these results indicate that the methylation pattern of CpG doublets is inherited on DNA replication.

Since the gene products (rRNA) contain considerable numbers of methyl groups it might be asked whether the methylation pattern of rRNA resembles that of rDNA. The answer is no. The methylation patterns of several vertebrate rRNAs including *Xenopus* have been examined in detail (Khan *et al.*, *Biochem. J.* **169**, 531; 1978). In contrast to the specific CpG methylation pattern of rDNA, rRNA is methylated principally on 2' hydroxyl groups of ribose moieties in a wide variety of specific primary sequences. The data on rDNA methylation have provided a much more detailed structural view of this phenomenon than anything hitherto available in the DNA of higher organisms, even though no functional explanation for DNA methylation is immediately apparent. □

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Genes in pieces: were they ever together?

from W. Ford Doolittle

THE recent discovery that many eukaryotic structural genes are interrupted by stretches (sometimes very long) of non-informational 'intervening' DNA¹⁻⁷ is the latest and most dramatic demonstration of the striking differences between the organisation of prokaryotic and eukaryotic genomes. The assumption that the tightly organised prokaryotic genome is 'primitive' and represents the sort of organisation found in the common ancestor of the prokaryote and eukaryote genomes is so common that it is usually only implicitly stated⁸⁻¹⁶. However, there is no direct evidence in favour of this assumption and some indirect evidence against it. I would like to argue that the eukaryotic genome, at least in that aspect of its structure manifested as 'genes in pieces' is in fact the primitive original form.

Gilbert¹ has proposed that intervening DNA serves to speed evolution; the mature polypeptide chain is translated from a spliced mRNA derived from a primary transcript of both intronic (non-informational DNA) and exonic (coding) DNA. Occasional imprecise splicing can generate new proteins assembled from parts of old ones

without sacrificing the original genes. Novel combinations conferring advantage can be fixed by minor alteration in the (presumably intronic) splicing recognition sites. So eukaryotes can evolve new and complex functions more rapidly than can prokaryotes without needing to increase the rate of point mutational change in established sequences. Indeed rates of sequence change seem relatively constant and unrelated to rates of morphological diversification in many eukaryotic lineages¹².

How did this mechanism for promoting evolutionary change arise? Most molecular biologists assume that the eukaryotic genome arose from a 'simpler' and more 'primitive' prokaryotic genome rather like that of *Escherichia coli*^{9,11}. Most evolutionary biologists assume that this happened between 0.8 and 1.4 billion years ago¹³⁻¹⁵. The subsequent rapid diversification and apparently 'progressive' evolution of higher forms is thus seen as a corollary of progressive increase in genomic complexity⁹⁻¹¹, a process which must now include the insertion of intervening DNAs into previously intact structural genes.

However, when one comes to consider how this type of organisation could have arisen from a tightly-organised

genome similar to that of modern-day prokaryotes, considerable problems arise. It is extraordinarily difficult to imagine how informationally irrelevant sequences could be introduced into pre-existing structural genes without deleterious effects. Insertion sequences may play a part in prokaryote evolution but their introduction into unique essential genes is most often lethal¹⁶. Even if there had been some pre-existing RNA processing machinery serving some other function, which could render the inserted DNA innocuous, the cell would enjoy no immediate selective advantage from allowing such an insertion. The idea that cells would do so to increase their potential for future evolution is not a Darwinian one; it imputes purpose. Finally, there is now good evidence that eukaryotic and prokaryotic genomes diverged from each other more than 2.5 to 3.0 billion years ago¹⁷⁻¹⁹. This evidence derives from sequence comparisons of ribosomal RNAs from bacteria, methanogens and eukaryotes which suggest that the genomes encoding them diverged when the translational apparatus was still undergoing rapid, selected evolutionary change. If ribosomes were in flux, it is not unreasonable to suggest that genome organisation itself was then still undergoing rapid and selected evolution.

I would suggest that DNA replication and transcription as well as translation would probably have been unfaithful in the last common ancestor of prokaryotes and eukaryotic nuclei. Unfaithful systems can of course evolve rapidly and rapidity is required to account for the appearance of identifiable prokaryotic (blue-green algal) cells in the scant 1.5-2.0 billion years that micropalaeontologists now allow²⁰⁻²¹. However, unfaithful cells are not likely to have small tightly-organised genomes like that of *E. coli*; genetic information must be redundantly coded for any of it to be 'correctly' expressed²². In such cells, a genes-in-pieces organisation (with RNA splicing) could not only have its current (evolutionary) role¹, but an additional essential role: to ensure that transcripts of exons (reiterated but often incorrectly replicated and transcribed were at least occasionally assembled so as to template functional proteins. As replication, transcription and translation became more faithful, such insurance became less necessary and the replication and transcription of redundant and non-informational DNA became increasingly irrelevant and burdensome. There therefore was (and still is) at each generation pressure to eliminate these DNAs (and advantageous elimination of non-informational DNA is much easier to envisage than is harmless or advan-

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tageous introduction of such DNA). The benefit, at each generation, was increased efficiency, but the price was the loss of potential for further evolution, at a level above that of point mutational change.

The first to pay this price were the ancestors of modern bacteria; their streamlined genomes are organised and expressed with an efficiency surpassed only by that of the genomes of the viruses which infect them, but their morphological and functional complexity is limited. Lower protists, which show among themselves a variety of different approaches to genomic streamlining²³, and which may retain traces of a genes-in-pieces organisation²⁴, were perhaps next. There will of course have been at each stage cells which did not succumb to the selective advantage of genomic simplification, although they resisted such change accidentally, not purposively. The 'higher' forms are not more complex because they have recently increased genetic plasticity. They are, rather, 'higher' and more complex because their ancestors were, at each generation, those very organisms which

accidentally (and at some selective disadvantage to their immediate descendants) retained the genetic plasticity inherent in the genomes of the primitive ancestors of all cells. □

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Incorporation of uracil into DNA

from Gerard O'Donovan

WHY does DNA contain thymine and not uracil? Over the past few years mechanisms preventing the incorporation of uracil into DNA have been uncovered in bacteria but more recently, several groups have shown that if these constraints are circumvented an apparently functional DNA containing uracil can be produced.

The incorporation of uracil into DNA is prevented by at least two mechanisms. The first is the presence of dUTPase activity which hydrolyses dUTP to dUMP thereby keeping the endogenous dUTP pool low while providing the dUMP substrate for thymidylate synthetase (see figure). The second is the presence of uracil-DNA glycosidase activity which removes uracil from single and double-stranded DNA (Lindahl *Proc. natn. Acad. Sci. U.S.A.* **71**, 3649; 1974).

Viable dUTPase mutants (*dut* mutants) were isolated by Hochauer and Weiss (*Fed. Proc.* **35**, 1492; 1976). These mutants are identical with previously described *dnaS* and *sof* mutants. As expected these mutants incorporate uracil into their DNA (Tye *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 154; 1977). They also accumulate labelled transient 4-5S DNA fragments

after 5-10 s pulses of ³H-thymidine (Tye *et al. op. cit.*). These fragments result from the increased incorporation of uracil into DNA as a consequence of the dUTPase defect, followed by excision repair of the incorporated uracil, probably by uracil-DNA glycosidase.

Viable mutants deficient in uracil-DNA glycosidase (*ung* mutants) have been isolated by Duncan *et al. (Fed. Proc.* **35**, 1493; 1976). The next step was obviously to examine *dut ung* double mutants for uracil incorporation into DNA. Warner and Duncan have recently reported (*Nature* **272**, 32; 1978) the presence of ³H-uridine in the DNA of such mutants of *E. coli* and in the DNA of T4 phage propagated in them. Up to 30% of the thymine in T4 DNA seems to have been replaced by uracil and this DNA is functional as judged by the following findings.

When *ung*⁻ cells of *E. coli* are in-

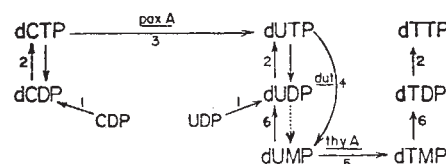
fectured they are killed equally well by the uracil-containing T4 phage DNA (T4-U) and the uracil-free phage (T4-T). The base composition of the total DNA of the infected cell and of the DNA packaged into the phage are the same. When the T4-U DNA is injected into an *ung*⁻ cell (unable to initiate uracil-specific degradation) the T4-U DNA is normally functional as evidenced by its ability to be replicated and to direct phage synthesis.

When T4-U phage DNA is injected into wild-type *E. coli* (*ung*⁺) the T4-U phage DNA is degraded so rapidly that it is not functional.

So why then does DNA contain thymine? Since the frequent random substitution of uracil for thymine during T4 DNA synthesis seems to have no serious consequences for the subsequent phage replication, the occasional misincorporation of uracil may have only minor effects on replication and transcription. Although it is believed to be attacking the A-U base pairs in the altered DNA the most important function of the uracil-DNA glycosidase normally *in vivo* therefore may not necessarily be to remove misincorporated uracil but to excise uracil produced by deamination of cytosine *in situ* by cytosine deaminase, thus preventing transition mutations. Warner and Duncan explain the importance of the presence of thymine rather than uracil in DNA by proposing that the absence of A-U base pairs allows specific recognition and excision of the potentially mutagenic mismatches produced by cytosine deaminase. Indeed Duncan has recently been able to show that *ung* mutations are mutagenic particularly when C→U transitions were specifically studied.

One final point seems pertinent. In a recent collaborative study Tye *et al. (Proc. natn. Acad. Sci. U.S.A.* **75**, 233; 1978) have shown that uracil persists in the DNA of the *dut*⁻ *ung*⁻ double mutant through several generations. The *dut*⁻ *ung*⁻ mutant seems unaffected by levels of uracil in its DNA up to 1 per 100 nucleotides. This finding, taken together with the 30% substitution in phage T4 DNA reported here, strongly suggests that uracil incorporation into DNA is a common phenomenon. Experiments are in progress to determine how much uracil can be substituted for thymine in phage DNA as well as in *E. coli*. As pointed out by Warner, T4 DNA has to interact with far fewer proteins than does the DNA of bacterial and eukaryotic cells. Thus it seems likely that cells will be rather less tolerant of thymine replacement by uracil than are viruses. □

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articles

9-(2-Hydroxyethoxymethyl)guanine activity against viruses of the herpes group

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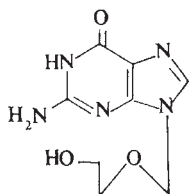
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Of a series of nucleoside analogues synthesised, 9-(2-hydroxyethoxymethyl)guanine was found to have marked antiviral activity in animal models of herpes virus infections, associated with very low toxicity.

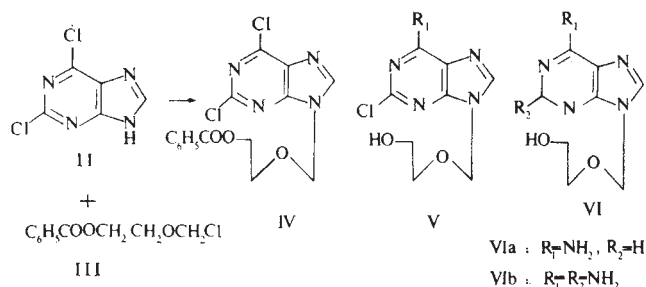
MUCH effort has been expended in the search for effective antiviral agents, but relatively few effective agents have been found. The clinically useful compounds are 1-methylisatin 3-thiosemicarbazone (methisazone)¹, 1-aminoadamantane hydrochloride², 5-iodo-2'-deoxyuridine (idoxuridine)³, 9-β-D-arabino-furanosyladenine (vidarabine)⁴ and trifluorothymidine⁵. We now report on a new compound with good antiviral activity and low host toxicity—9-(2-hydroxyethoxymethyl)guanine (I), which is also referred to as acycloguanosine.



Chemistry

In recent years several interesting nucleosides have been prepared which possess antiviral activity. These nucleosides are analogues of natural products in which chemical modifications have been made in the pyrimidine or purine heterocycle, in the carbohydrate moiety, or in both⁶⁻⁸. Because earlier studies had shown that the intact cyclic carbohydrate moiety was not necessary in order to mimic nucleoside binding to enzymes⁹, a programme was initiated to synthesise a range of nucleoside analogues in which the cyclic carbohydrate moiety was replaced by an acyclic side chain attached to the heterocyclic base by a glycosidic linkage. The initial synthesis of this class of compounds focused on the key intermediate 2,6-dichloro-9-(2-benzoyloxyethoxymethyl)purine (IV), which was prepared in 41% yield by the condensation of II and III in *N,N*-dimethylformamide in the presence of triethylamine as the acid acceptor. Because of the differences in the chemical reactivity of the 2- and 6-positions in IV, selective substitution of the 6-chloro group could be accomplished as well as simultaneous deblocking of the acyclic side chain to give V. Thus, when IV was allowed to react with methanolic ammonia at 85 °C, a good yield of V ($R_1 = \text{NH}_2$) was obtained. Treatment of V ($R_1 = \text{NH}_2$) with nitrous acid, followed by reaction of

the resultant deaminated product with methanolic ammonia to displace the 2-chloro group gave a moderate yield of 9-(2-hydroxyethoxymethyl)guanine (I) for the two-step sequence. By a related sequence of reactions the syntheses of VIa and VIb were accomplished. Thus, by the use of this synthetic



procedure, a wide variety of compounds with different substituents at the 2- and 6-positions of the purine nucleus has become available.

Antiviral activity

Preliminary detection of antiviral activity was carried out *in vitro* by means of plaque inhibition tests^{3,10} with monolayers of Vero cells infected with the ICI strain of type 1 herpes virus. Activity was measured using plaque reduction assays in the same virus-cell system, in which the test compounds were present in the agarose overlay in an appropriate series of doubling concentrations.

The plaque counts were expressed as a percentage of the number obtained in similar control cultures, and were plotted against the logarithm of the concentration of compound to give dose-response lines from which the ID_{50} values were calculated. Several standard anti-herpes compounds were assayed in a similar manner, and the ID_{50} values obtained are shown in Table 1, together with the relative potencies in comparison with idoxuridine, the compound most frequently used in clinical practice. Compound VIb is somewhat more active than vidarabine, but its activity is greatly exceeded by the guanine analogue (I), which is 160 times more active than vidarabine and 10 times more active than idoxuridine. Compound I also had a similar degree of activity ($\text{ID}_{50} = 0.14 \mu\text{M}$) in plaque reduction assays with type 2 strains of herpes, whereas the standard compounds generally showed lower activity. Furthermore, I has extremely low toxicity against uninfected Vero cells, which remain viable in concentrations up to 20 mM, the limit of solubility.

Preliminary studies showed that compound I inhibits the multiplication of varicella-zoster virus, cytomegalovirus and

Table 1 Relative potencies of 9-(2-hydroxyethoxymethyl)guanine and some standard anti-herpes compounds

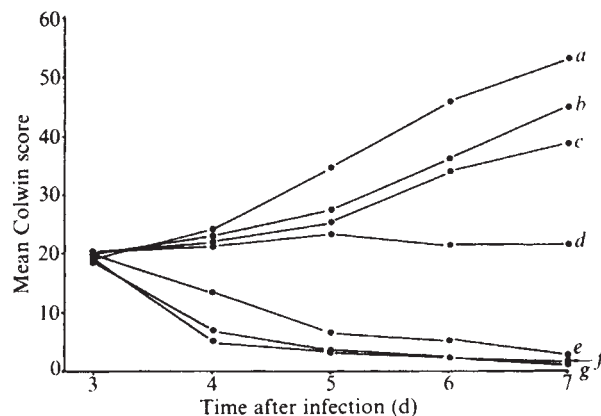
Compound	ID ₅₀ (μM)	Potency relative to idoxuridine (= 100)
Phosphonoacetic acid	57.5	1.7
VIa	40	2.5
Vidarabine	16	6
VIb	10.5	9.5
Trifluorothymidine	1.5	67
Idoxuridine	1	100
Cytarabine	0.2	500
Acycloguanosine (I)	0.1	1,000

B virus (K. R. Dumbell and E. A. Boulter, personal communications) but has no effect on vaccinia virus, adenovirus type 5 and a range of RNA viruses comprising rhinovirus 1B, Mengo, Semliki Forest, Sindbis, Bunyamwera, yellow fever, measles, respiratory syncytial virus and the NWS strain of influenza virus.

The compounds were also tested for activity *in vivo* in mice infected intracerebrally with type 1 herpes virus. Infected mouse brains were ground in a mortar and suspended in a mixture of phosphate-buffered saline and glycerol, and the stock suspensions thus obtained had LD₅₀ titres of around 10⁵. Groups of 10 mice were infected intracerebrally with 0.025 ml of stock suspension diluted to give doses of around 3 and 300 LD₅₀.

Test compounds were administered twice daily for 5 d by the oral route in a dose of 100 mg per kg body weight. Similar groups of infected mice were left untreated as controls. Survival times were recorded to the nearest half day over an observation period of 14 d. The results obtained in typical experiments with VIb and I are shown in Table 2. With both compounds the survival time was considerably prolonged at both dose levels of virus, and some mice in the treated groups survived the period of the experiment without showing any signs of illness. Similar results were obtained when the compounds were administered subcutaneously (s.c.).

Acycloguanosine and VIb were also evaluated against experimental herpetic keratitis in rabbits. Both corneas of a rabbit were infected with type 1 herpes virus by means of a microtrephine. The method used was a modification of that described by Jones *et al.*¹¹. After 3 d, when ulceration at the sites of infection was well developed, one eye was treated and the other was left untreated as a control. Acycloguanosine was administered five times at intervals of 2 h during the working day as an ointment prepared in sterile ophthalmic petrolatum ointment base. Treatment was continued for 5 d. Each day fluorescein was instilled and drawings were made of the extent of the ulceration in both eyes, and the various signs of infection were given numerical scores according to the method of Corwin *et al.*¹². In the untreated eye ulceration progressed until the lesions became confluent. At this stage the eye was grossly inflamed, with injection of the conjunctiva, purulent discharge,

**Fig. 1** Treatment of herpetic keratitis in rabbits with ophthalmic ointments containing various concentrations of acycloguanosine. a, Control; b, 0.03%; c, 0.1%; d, 0.3%; e, 1.0%; f, 2.0%; g, 3.0% acycloguanosine. Treatment was begun on day 3.

opacity of the cornea and extensive ulceration. In the treated eye the extent of the ulceration regressed after the first day of treatment, and by the 5th day healing was complete, apart from some residual injection of the conjunctiva. The mean Corwin scores recorded in experiments with ointments of different concentrations of acycloguanosine carried out on a total of 60 rabbits are given in Fig. 1, which shows that this compound produces a satisfactory cure of herpetic keratitis in concentrations of 1% or higher. Similar results were obtained with aqueous solutions of VIb in concentrations of 1% and higher.

The effect of the compounds on cutaneous herpes infections was studied in guinea pigs. Both flanks of the animal were shaved and further epilated with a barium sulphide preparation. The skin was then washed and dried, and 4 h later a suspension of type 1 herpes virus containing 10⁶ plaque-forming units (PFU) per ml was inoculated at eight sites on both flanks by scarification. After 18 h, when lesions had begun to develop, those on one flank were treated twice daily with a water-soluble ointment formulation of acycloguanosine, and the lesions on the other flank were left untreated as a control. Treatment was continued for 3 d and the lesions on both flanks were photographed. The results of treatment with a 1% ointment of the compound are shown in Fig. 2. On the treated side the herpetic lesions have healed, the skin showing only residual trauma from scarification, whereas on the control side the lesions are in mid-course of evolution. Satisfactory cures were also obtained with VIb and acycloguanosine as a 1% solution in dimethylsulphoxide.

Metabolic disposition

Studies have been carried out to evaluate the absorption, metabolism, tissue distribution and excretion of 9-(2-hydroxyethoxymethyl)guanine in several animal species.

To evaluate the urinary and faecal excretion ¹⁴C-acycloguanosine labelled at the 8-position of the guanine nucleus was given to mice either orally or subcutaneously. Absorption of the drug given by mouth was incomplete. In the 4-h urine sample 23% of the ¹⁴C was recovered. In the 24- and 48-h urine samples less than half the given dose was recovered. Most of the remaining radioactivity was found in the faeces. In mice treated s.c. with ¹⁴C-acycloguanosine (50 mg per kg body weight; 5.5 × 10⁷ d.p.m. per mouse) 82% of the radioactivity was excreted in the urine by 4 h and 94% by 24 h; the faecal excretion was 2.3%. Over 95% of the radioactivity excreted in the urine over a 24-h period was recovered as the unmetabolised compound. Analysis of the urine by high-pressure liquid chromatography (HPLC) on an Aminex A-28 column eluted with 15 mM sodium acetate buffer, pH 4.1, showed a single major peak of ¹⁴C radioactivity with a retention time of 50 min, corresponding to authentic acycloguanosine. All fractions from

Table 2 Treatment of herpetic encephalitis in mice with VIb and 9-(2-hydroxyethoxymethyl)guanine (I)

Compound	Virus dose (LD ₅₀)	Group*	Survival time (d)									
VIb	3	T	8	8	12	12	14	14	S†	S	S	S
		C	6	6	7	7	8	8	8	8	9	9
	300	T	4	4	6	6	7	7	7	7	7	8
		C	3	3	3	3	3	3	3	3	3½	3½
I	3	T	8	8	8½	10	14	14	S	S	S	S
		C	6	6	7	7	8	8	8	8	9	9
	300	T	6	6	6	7	7	7	7	8	8	8
		C	3	3	3	3	3	3	3	3	3½	3½

Data are shown for 10 individual animals per group.

*T, treated; C, control.

†S, survived with no signs of illness.

the column were counted. There was practically no cleavage of the compound *in vivo*, since only traces of radioactive guanine and uric acid were found in the urine. A minor metabolite (about 1% of the radioactivity) which was highly retained on the liquid chromatography column was characterised as 9-carboxymethoxymethylguanine by comparing its retention time (390 min) with that of the synthetic compound.

To determine whether repeated administration of acycloguanosine to mice would increase its biotransformation, the drug was given daily for 7 d to a group of mice (50 mg per kg, s.c.). On the last day the mice received a dose of labelled compound. The HPLC profile of ^{14}C radioactivity in the 24-h urine of mice showed no alteration in the metabolism of the compound.

Administration of ^{14}C -acycloguanosine (50 mg per kg, s.c.) to groups of CD-1 mice gave the tissue distribution results shown in Fig. 3. In all tissues there was a rapid disappearance of the compound in the first 2 h after administration, followed by a slower disappearance over a 24-h period. The levels in the kidney, intestine, lung and liver were higher than in the blood. The concentration in the brain was significantly lower than in the blood and other tissues, but the compound was still detectable at 24 h. When ^{14}C -labelled compound was administered orally (50 mg per kg) to mice its concentration in the plasma at 30 min was $13.5 \text{ nmol ml}^{-1}$, but its disappearance from the plasma was slower than in the case of subcutaneous administration. There was no appreciable binding of acycloguanosine to plasma or to any tissue constituents. In an *in vitro* study of plasma binding ^{14}C -acycloguanosine was incubated with the plasma of CD-1 mice at 37°C for 45 min in a Dubnoff shaking incubator. Plasma binding was determined by ultrafiltration.

Fig. 2 Treatment of cutaneous herpes infection in a guinea-pig with 1% acycloguanosine ointment in a water-soluble base. a, Treated side; lesions healed after 3 days of treatment. b, Control side; lesions still in process of development.

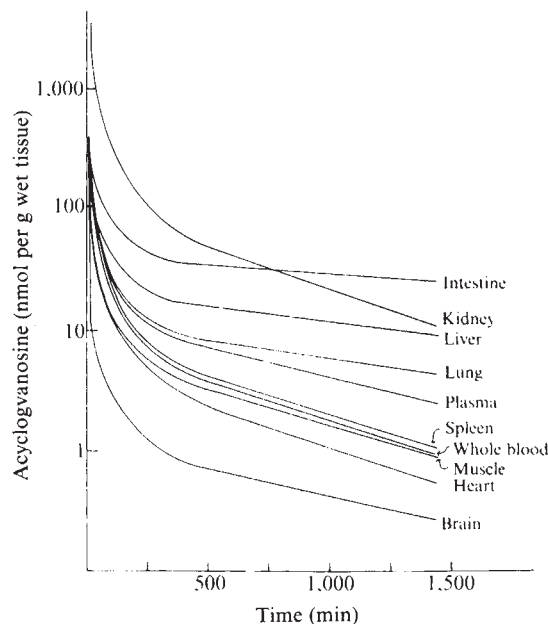
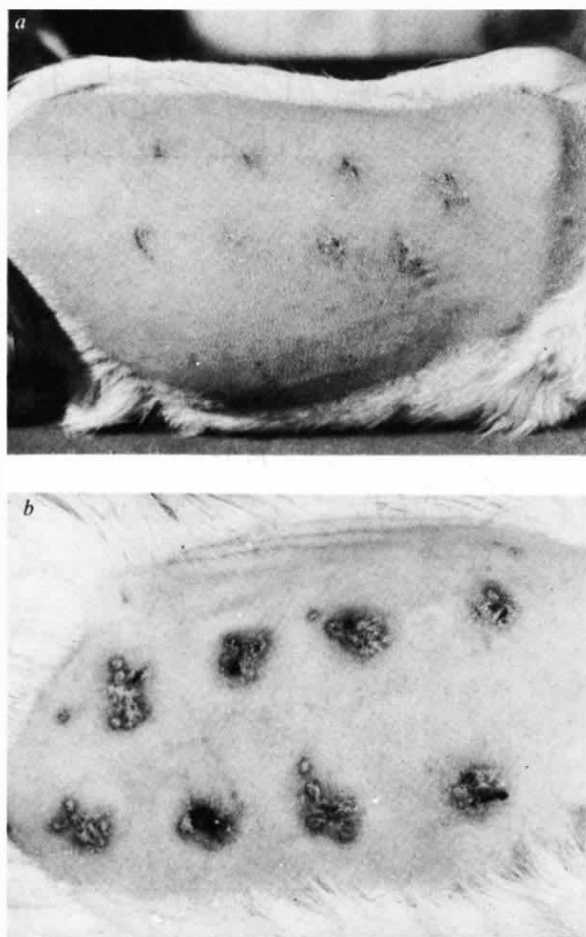


Fig. 3 Concentrations of acycloguanosine in mouse tissues determined from concentrations of ^{14}C in these tissues at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h after s.c. administration of 8- ^{14}C -acycloguanosine, 50 mg per kg, (specific activity $4.5 \text{ mCi mmol}^{-1}$) to groups of 3 mice. Curves were fitted to the experimental points by a computer program. Because the drug is not catabolised to any extent, the ^{14}C concentration corresponds to drug concentration.

At a drug concentration of $12 \mu\text{M}$ in the plasma no binding was observed. When the drug concentration was $88 \mu\text{M}$, 12.7% of the drug was bound to plasma.

In studies of the acute toxicity of acycloguanosine, the LD_{50} in mice given the drug orally was greater than 10,000 mg per kg whereas the LD_{50} in mice by the intraperitoneal route was 1,000 mg per kg. In 30-d studies in mice daily oral administration of 450 mg per kg of acycloguanosine produced no toxicity.

Related studies in rats and dogs on the metabolism, pharmacokinetics and toxicology of acycloguanosine will be described elsewhere; preliminary results are similar to those found in mice.

A study of the mechanism of the selective action of acycloguanosine has shown that it is phosphorylated by the herpes-specified thymidine kinase and converted to the triphosphate in herpes-infected cells to a much greater extent than in uninfected cells. Moreover, the triphosphate of acycloguanosine is more inhibitory to the viral DNA polymerase than to the α -DNA polymerase of the cell¹³.

The higher antiviral activity in comparison with existing anti-herpes compounds, good activity in animal models, the very low toxicity and the lack of metabolic degradation on systemic administration make 9-(2-hydroxyethoxymethyl)guanine a promising candidate for clinical trials against the various manifestations of herpesvirus infection in man.

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Prediction of chain turns in globular proteins on a hydrophobic basis

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Peptide chain turns are those parts of a globular protein where the backbone changes its direction. A simple, non-empirical hypothesis is presented to account for both the turns and the hydrophobic core of the protein using only the amino acid sequence.

KAUZMANN'S generalisation¹ that globular proteins have a hydrophobic core forms the basis of our ideas about protein tertiary structure. In this article that generalisation is extended and used to understand the hydrophobic basis of peptide chain turns. Subsequent publications will deal with other aspects of secondary and tertiary structure.

Peptide chain turns are those parts of a globular protein where the backbone is folded back on itself; they are in effect changes in the overall direction of the polypeptide chain. Different investigators have used the concept of a turn in qualitatively differing ways. Turns in the general sense, as used here, were first described by Kuntz², Lewis *et al.*³ and Crawford *et al.*⁴, but significant elements of this concept are seen in earlier work of Dickerson *et al.*⁵, Blake *et al.*⁶, Venkatachalam⁷ and Kendrew⁸.

Although the concept of a chain turn seems intuitively obvious on visual examination of a physical model of a protein, the lack of an objective structural definition has resulted in variant catalogues of turns compiled by different investigators for the same protein. For this reason, Rose and Seltzer⁹ devised an algorithm to identify turns from X-ray-elucidated coordinates. This algorithm treats the polymer chain as a curve in space and computes a discrete radius of curvature for that curve. A turn corresponds to a locus where the chain direction vector is changing rapidly and the value of the radius of curvature is at a local minimum. Although turns are shown to correspond to local minima in the radius of curvature, the correspondence is not 1:1; that is, a small fraction of the minima is not associated with turns, and additional analysis was developed to identify these loci. α -Carbon coordinates are the only information required by the algorithm, and the existence of hydrogen bonds at turn loci is irrelevant to the geometrical nature of the procedure. In this sense, the algorithm provides an objective criterion for the recognition of turns as strictly structural components in proteins.

Rose and Wetlauffer¹⁰ later showed that the number of turns in a globular protein is a linear function of the molecular weight, and this relationship was interpreted to mean that turns are determined by linearly local sequences of amino acids along the polypeptide chain.

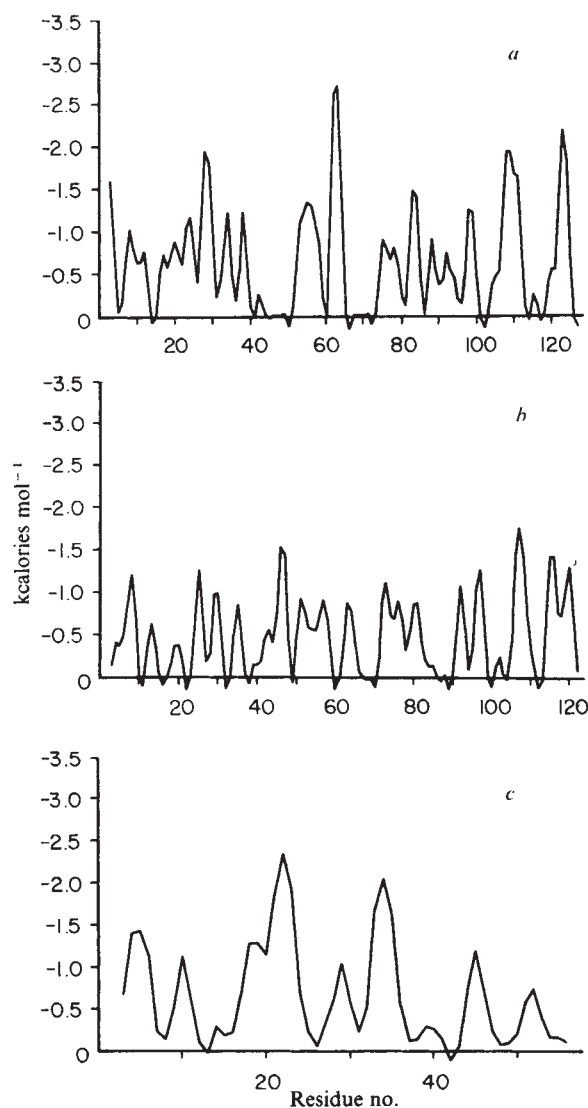
Hypothesis for turns

It is hypothesised here that turns occur at those sites in the polypeptide chain where the hydrophobicity is at a local minimum. A correspondence between turns and minima in hydrophobicity is not too surprising, for it has been observed repeatedly since Kuntz's work² that the sequence composition at turn sites is comparatively polar. Such an observation is implicit in the published 'predictive' schemes for finding turns^{11,12} and β bends⁷ (which are a subset of the turns). These predictive strategies usually proceed by seeking a

correlation between local collections of certain amino acids and a structural preference for turns, and in these schemes the observed turn-forming residues are all non-hydrophobic. Such an approach is almost entirely empirical, and prediction is about 70% successful at best¹³. In contrast, the hypothesis that turns correspond to minima in hydrophobicity has a chemical rationale and better predictive capability.

A method for locating the turns specified by the turns hypothesis is presented here. Linearly local clusters of large hydrophobic residues can be identified by scanning along a

Fig. 1 Smoothed hydrophobicity profiles for lysozyme (a), ribonuclease (b) and pancreatic trypsin inhibitor (c). A hydrophobicity profile plots the Nozaki and Tanford free energy of transfer against the residue number. Local minima on this curve correspond to the peptide chain turns.



protein's amino acid sequence. These clusters consist of from one to a few nearly consecutive residues in the sequence. Large hydrophobic residues are amino acids with hydrophobic side chains larger than alanine; they include tryptophan, phenylalanine, tyrosine, leucine, isoleucine, valine and methionine. In this approximate partitioning, adjacent clusters are separated from each other by a section of chain that is devoid of large hydrophobic groups. It is apparent from the three-dimensional structure of X ray-

elucidated proteins that groups of clusters are brought into proximity, thereby kinking the intervening chain. These kinks will occur at the comparatively non-hydrophobic sites along the chain, and they become the peptide chain turns; while the interactions of proximate hydrophobic clusters establish the apparent hydrophobic core of the protein. Using this strategy for partitioning the chain, it is possible to locate most of the turns merely by looking at an amino acid sequence.

Table 1 Comparison of turns

Lysozyme	Measured	Predicted	Levitt-Greer	Measured	Predicted	Levitt-Greer	Measured	Predicted	Levitt-Greer
1.	5	5.5		4.	19.4*		22.	110.0*	113
2.		(10.4)		5.	25.8	26-27	23.	117.9	116-119
3.	15	14.4		6.	31†		24.	120-124	121.2
4.	18-19	18.2	18-22	7.	37.6	38-39	25.	(127.7)	
5.	21	21.5		8.	42.2		26.	135-136	134.2
6.	24-26	25.8		9.	48.5		27.	142	142.8
7.		(31.4)		10.	55.2*	56			137-141
8.	35-36	36.0	36				Subtilisin		
9.	38	40.7	41	Flavodoxin			1.	11	9.3
10.	48	45.0, 50.0	48	1.	8.9	8-9	2.	13.3*	11
11.	55-56§		55-56	2.	(12)		3.	19-20	18.4
12.	62	59.6	62-64	3.	(16)		4.	26-28	23.6
13.	67	65.9		4.	(21)		5.	32	33.0
14.	70	72.1	67-71	5.	26-27		6.	40	38.5
15.	78	76.9‡		6.	31.4		7.	45-46	47.1
16.		80.5‡		7.	36	35-37	8.	53	54.6
17.	86	86.0	86-87	8.	41.2*		9.	57-58	57
18.		90.2*		9.	44-45†		10.	61-62	61-62
19.		(95.6)		10.	47	45	11.	(65.1)	
20.	102	101.8	101-106	11.	58	58-59	12.	72	70.3
21.	109-110	113.8‡		12.	64-65		13.	79	77.1
22.	117-118	117.3		13.	(67.3)		14.	84	
23.		126.8*	126	14.	(71.8)		15.	87	87.2
Ribonuclease				15.	75-76	76	16.	95-96	93.5
1.	3-4	5.0		16.	79		17.	99	98.7
2.		(10.7)		17.	90	89-90	18.	103-104	101.2
3.	14	16.1		18.			19.	(109.7)	
4.	18¶		17-18	19.	(101.4)		20.	117	117.4
5.	22	22.2	22	20.	106-107		21.	125	124.0
6.	24-25	27.3		21.	112	112-113	22.	120	129.2
7.	33-34	32.3		22.	119	119-120	23.	132-133	131.0
8.	36	37.7	37-40	23.	123	122-123	24.	(136.7)	
9.	38	40.1		24.	(133.1)	137	25.	(140.7)	
10.	42	43.6		Staphylococcus nuclease			26.	145-146	145.3
11.	50-51	48.9		1.	5-6	3-7	27.	155	155.8
12.		(54.2)		2.	9.9*		28.	161	161.8
13.	58-59	60.2	59-60	3.	15		29.	169	168.9
14.	62-63†		64	4.	20		30.	172	172.6
15.	67	67.7	66-67	5.	28-29	28-29	31.		178.1*
16.	70	69.5		6.	38-40	42	32.	183	183.0
17.	77	74.7	77	7.	45	44-46	33.	188-189	185.2
18.	80	78.2		8.	48	48-53	34.	194	194.0
19.	88	86.8, 89.0	88	9.	53-57		35.	201	201.3
20.	93	94.3	92-93	10.			36.		206.9*
21.		99.8*		11.	(56.9)		37.	211	211.4
22.	104	103.6		12.	(63.3)		38.	215-216	215.3
23.	108-109†			13.	70		39.	218-220	219.6
24.	113-114	112.2	112-114	14.	79	78-80	40.	(224.7)	
25.		117.7*		15.	81-82		41.	(229.8)	
26.		121.5*		16.	85	84-85	42.	240	237.9
Trypsin inhibitor				17.			43.	242-243	243.7
1.	5	7.8	8-9	18.	90-91		44.	(247.9)	
2.	13-14	12.7	12-13	19.	95	96	45.	252	253.5
3.	16	15.3		20.	100		46.	258	259.0
				21.	107		47.	261-262	265.0
							48.		271.5*
									270-274

Measured turns were found using the algorithm of Rose and Seltzer⁹ that identifies turns from structural criteria only. Predicted turns are the local minima in hydrophobicity. Levitt-Greer turns¹⁸ are the output of another algorithm that identifies reverse turns from structural criteria. Taking the measured turns as a standard, there are three ways the predicted turns can fail to be in agreement. (1) Overprediction: predicted turn does not correspond to any measured turn. (2) Underprediction: measured turn does not correspond to any predicted turn. (3) Alternate prediction: measured turn and predicted turn parse the chain differently. In cases of disagreement between measured and predicted turns, an α -carbon bent-wire physical model was used to help clarify the discrepancy. When visual inspection of the model affords a plausible explanation of the discrepancy, the turn is labelled with an asterisk, dagger or double dagger. These cases are discussed further in the text.

* Plausible overprediction: physical model reveals a believable alternate parsing that places an additional turn at this site.

† Plausible underprediction: physical model reveals a locally distorted helix or β strand in which the two pieces at either side of the local perturbation are almost colinear.

‡ Alternate prediction: measured turns and predicted turns both appear acceptable on strictly structural grounds in the physical model. Alternate parsings occur frequently around 3_{10} helical turns.

§ Turn not at solvent accessible surface of the molecule. Hydrophobic minimum fails to predict this turn.

¶ Algorithm was applied to the atomic coordinates for RNase-S.

To refine this analysis, a measure of the hydrophobicity of the amino acid side chains is needed, and this is taken to be the Nozaki and Tanford¹⁴ free energy of transfer from water to an organic solvent. With this information, a graph of residue number against hydrophobicity can be constructed easily for any protein. This protein hydrophobicity profile is then smoothed by taking a five-point moving average in place of the individual values of hydrophobicity. Any smoothing technique should suffice here; I have used least squares fitting to a quadratic polynomial according to a method of Savitzky and Golay¹⁵.

The smoothed hydrophobicity profile for three proteins is shown in Fig. 1. Use of the free energy of transfer allows each point in the sequence to be associated with its appropriate hydrophobic energy.

The hydrophobicity profile can now be differentiated to find local minima. A complete set of local minima for six proteins is listed in Table 1, including the three proteins of Fig. 1. This set of six was used to correspond to the examples in the report⁹ giving a structural basis for the recognition of turns. However, a set of approximately two dozen proteins was analysed and the results were validated by the use of physical models. The prediction works equally well in all cases.

It must be acknowledged that one or more local minima in hydrophobicity are always found to be associated with helical secondary structure. Of course, the peptide chain does change direction at the endpoints of a helix and local minima would be expected there. However, additional minima may occur between the ends of helices, and in these cases it might seem that the proposed solution overpredicts turns. Because a helix is comprised entirely of turns, the structural effect of a local minimum in hydrophobicity is masked when embedded within residues that promote a helix. For this reason, turns in Table 1 that are interior to a helix are given in parentheses. In a pragmatic sense, this means that practical prediction of the apparent turns also requires a knowledge of the helical residues. This is to say, apparent turns correspond to the set of local minima in hydrophobicity minus any minima that are at the interior of helices.

Empirical correlations usually avoid the problem of ostensible overprediction by first dealing with the helices, and then removing the predicted helical residues from further consideration before the prediction of turns. The relationship between hydrophobic minima and helices will be dealt with in another publication in which a method for predicting the helices will be presented. (In the six proteins in Table 1, 'in-helix' minima represent 14% of the total turns predicted.)

Identification of turns

A standard for comparison is needed to judge the merit of the set of predicted turns. Table 1 compares the predicted set and the results of the algorithm⁹ which locates turns on a strictly structural basis. Although both the Rose-Seltzer algorithm and the minima in hydrophobicity give rise to discrete points as the loci of turns, it is not sterically possible for the polypeptide backbone to execute a turn within a single residue. For example, the subset of turns known as β bends extends over four consecutive residues¹, and very gentle turns involving up to eight residues have been observed². In general, a physical turn occupies an interval $I=[a,b]$ where a is the first residue involved in the turn, b the last residue, and $(b-a)\geq 4$. The turn locations cited in Table 1 should be viewed as internal to such an interval. For this reason, a difference of two or three residues between measured and predicted values in Table 1 need not be critical. It is reassuring that the number of hydrophobic minima compares favourably with the number of measured turns, excluding minima within the helix.

As mentioned above, there is no certified list of turns for

a protein. Indeed, the absence of such a list prompted development of the Rose-Seltzer analysis. In any analysis, turns are ultimately a property of the protein's three-dimensional geometry, and they must be apparent on visual inspection of a physical model. It should be emphasised that of the order of 15% of the turns are ambiguous in any protein model, and this limitation constrains the optimum correspondence that can be expected between any two methods for locating turns from strictly structural criteria. Ambiguity arises when the backbone experiences frequent changes of direction within a small number of residues. For example, the S turn in lysozyme from residues 17-22 can be construed as having either two turns (at residues 17 and 22) or three (at residues 17, 20 and 22); in the absence of additional information, either alternative is acceptable. Ambiguity also arises when there is more than one way to parse the backbone. Again in lysozyme, residues 109-110 can be viewed as a turn, but deferring the turn location to residue 114 is equally acceptable on structural grounds. In this latter example, placing the turn at residue 114 seems preferable because residue 110 is within a 3_{10} helix, but this preference is due to chemical, not structural criteria.

In addition to the comparison made in Table 1, the predicted turns were compared with α -carbon bent-wire backbone models^{16,17}, and where the algorithmic measure and the hydrophobic minimum are not in accord, a model was used to clarify the discrepancy. This visual comparison usually suggested an acceptable alternative way to parse the chain at these loci. Moreover, visual inspection showed that the turns prediction hypothesis is consistently more sensitive to the secondary structure of the protein than the structural algorithm. Sensitivity to secondary structure is demonstrated by resolving instances of multiple parsings in favour of a choice that avoids situating the turn at the interior of some other evident secondary structure, as, for example, in the case of residue 114 in lysozyme. Although the Rose Seltzer algorithm deliberately excludes secondary structure as an analytical criterion, the sensitivity to secondary structure shown by the turns hypothesis indicates that some of the hydrogen bonding is an implicit consequence of the local chain hydrophobicity.

Using the structural algorithm as a standard, 117 turns are identified for the six proteins in Table 1, and there are 26 discrepancies with non-helical minima. In the worst case, this amounts to 78% agreement between measured and predicted turns at non-helical loci. However, visual inspection of bent-wire models reveals that most of these discrepancies are due to a plausible alternate parsing of the chain. Because plausible alternate parsings inevitably arise from the inherent ambiguity in locating turns on strictly structural grounds, 22 of the discrepancies in Table 1 should not necessarily be counted in error. Thus, in the best case, the agreement between measured and predicted turns is 97% at non-helical loci.

From the foregoing discussion, it seems that local minima in hydrophobicity give rise to a set of turns that is as authentic as the set identified on structural grounds from X-ray coordinates. The evidence strongly supports the proposed hypothesis for turns.

Comparison with other algorithms

As mentioned above, the literature contains various definitions of turns, and these discrepancies complicate possible comparisons. Most often, investigators have used β bends¹ as a paradigm for recognising the sharply defined reversals in chain direction, known as reverse turns; consequently these procedures can fail to identify the more gentle turns. Such a constraint was deliberately not built into the Rose-Seltzer algorithm, and it has no obvious relationship to the hypothesis for turns. For this reason, our structural algorithm ought to identify a set of turns that includes reverse-turns as a proper subset, and the turns hypothesis

ought to predict a set of turns that also includes any reverse turns.

In the following paragraphs, some comparisons are made between the results of the turns prediction hypothesis and an alternate algorithm for finding reverse turns from X-ray coordinates. In addition, the turns hypothesis was applied to the amino acid sequence of adenylyl kinase in order retroactively to participate in an ingenious comparison sponsored by Schultz¹¹, who withheld the X-ray structure of this molecule to provide an unbiased test of the accuracy of procedures for prediction. Any comparison of the turns hypothesis with a method that reveals only the reverse turns is necessarily incomplete, however, because reverse turns are a proper subset of total turns and so possible overprediction cannot be detected. In compensation, however, reverse turns are, by definition, more sharply defined than gentle turns, and the inherent ambiguity that engenders alternate parsings is greatly reduced for this category of turns.

Levitt and Greer¹⁸ published an algorithm for identification of secondary structure from X-ray coordinates. Their criterion for turns was meant to identify only the reverse turns which should be a subset of the turns given by the Rose-Seltzer algorithm. The Levitt-Greer reverse turns for the six proteins used in this study are listed in Table 1.

Of the 70 reverse turns identified by the Levitt-Greer algorithm for the six proteins in Table 1, five of these (or 7%) are not found at all by the Rose-Seltzer algorithm. However, four of the five are final turns within a few residues of the C terminus of the chain where the two structural algorithms disagree due to chain-end effects. With these end effects set aside, only one of the Levitt-Greer reverse turns is missed completely by the Rose-Seltzer algorithm. In addition, three of the turns (4%) found by the Levitt-Greer algorithm are situated between three and four residues away from the corresponding turn identified by the Rose-Seltzer algorithm; these three cases correspond to alternate parsings of the chain.

Taking the Levitt-Greer algorithm as an alternate standard with which to judge the merit of the set of predicted turns, there are at worst six discrepancies (91% agreement) with the set of measured turns. Of these, two turns identified by the structural algorithm are missed completely by the turns prediction hypothesis; one turn is missed because the structural algorithm was applied to RNase-S; and there is one instance each of plausible overprediction, plausible underprediction and alternate prediction as described in Table 1. This amounts to a best case agreement of 97% between turns identified by the Levitt-Greer algorithm and the corresponding turns predicted by the turns hypothesis. Overprediction by the turns hypothesis cannot be scored in this comparison of course because the Levitt-Greer algorithm identifies only a subset of the total turns.

Comparison between the results of the hypothesis for turns and the literature on empirical correlations is also beset by a lack of congruency in the definitions of turns. For example, Schultz¹¹ withheld the X-ray-elucidated details of adenylyl kinase until various groups had an opportunity to record their secondary structure predictions for that molecule. Comparisons of both the predicted and the experimentally determined results were then collected and published.

The X-ray data for adenylyl kinase were derived from a 3-Å electron density map and, consistent with resolution at this level, the only bends identified by Schultz are those where the backbone changes in overall direction by more than 120°. Schultz found ten bends that satisfy this restriction. These are extracted from the bar graph in Fig. 1 of his paper¹¹ and shown here in Table 2.

In comparison, the Levitt-Greer algorithm to identify bends from X-ray coordinates defines as reverse turns those loci where the backbone changes in overall direction by

more than 90°, as measured by their α angle. Thus the Levitt-Greer algorithm ought to find a superset of bends with respect to the more restrictive criterion used by Schultz. Of the ten bends identified by Schultz, the Levitt-Greer algorithm finds eight of these (80% agreement) and four additional reverse turns as well.

Results of the turns prediction hypothesis are also shown in Table 2. All ten of the Schultz bends are successfully predicted by the hypothesis (100% agreement). Of the four additional reverse turns in adenylyl kinase identified by the Levitt-Greer algorithm, three of these are also predicted by the turns hypothesis, while a turn corresponding to the fourth is predicted to occur three residues away. This amounts to a worst case agreement of 92% between the Levitt-Greer reverse turns and the turns prediction hypothesis, and a best case agreement of 100% if the turn at residues 66-67 is construed to be an alternate parsing. Once again, the predicted turns are a superset of the measured turns, and there is no way to score for possible overprediction.

Table 2 Comparison of measured and predicted turns for adenylyl kinase

Schulz	Levitt-Greer	Predicted
16-23	16-19	16.6, 20.0, 22.3
29-32	32	30.4
		35.6
38-42		39.6
48-52		49.6
		54
62-65	62-64	63.4
	66-67	70.4
84-88	84-86	87.2
		93.4
	96-98	97.2
		107.1
109-112	110-112	111.6
		122.4
134-137	137-140	136.3
		143.3
	158	157.8
166-169	167-168	166.5
		171.6
175-178	177-178	176.5
	193	191.3

Turns in column 1 are taken from Fig. 1 of the comparison by Schultz¹¹. Turns in column 2 are taken from Table 10 of ref. 18. Turns in column 3 are local minima in hydrophobicity as specified by the turns prediction hypothesis, but excluding those local minima that are interior to a helix.

It should be noted that the turns prediction hypothesis is quite insensitive to any uncertainties in the free energy of transfer used to measure the chain hydrophobicity. Indeed, prediction is almost as successful when the side chains are designated as either nonpolar or polar and assigned $\Delta G_{\text{transfer}}$ values of -1 and 0, respectively.

Persistence of local structure

Local maxima in hydrophobicity are also evident in Fig. 1, and the loci corresponding to these peaks appear to fold relative to each other to establish the tertiary structure and the hydrophobic core of the protein. Examination of distance plots¹⁹ confirms that the tertiary interactions (which appear as off-diagonal contacts in a distance plot) in the protein correspond to peaks in the hydrophobic profile and, conversely, that every peak is utilised at least once in a tertiary interaction. As mentioned earlier, interactions between hydrophobic maxima are presumed to be responsible for the occurrence of chain turns at the intervening sites where hydrophobicity is at a local minimum.

I have argued here that local minima in hydrophobicity provide a sufficient chemical basis for understanding the occurrence of peptide chain turns. The fact that these

short, linearly local sequences of amino acids are still discernible as turns in the three-dimensional structure has important implications for protein folding. In an earlier article, a structural segment was defined as a continuous sequence of linear chain neighbours bounded by consecutive chain turns⁹. I have shown here that linearly local interactions suffice to partition the polypeptide chain into its structural segments and turns, and that these locally defined moieties persist in the product of the folding process. The persistence of local structure is the basis of our model of protein folding²⁰.

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DRB-induced premature termination of late adenovirus transcription

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Exposure of HeLa cells to 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) late in the course of adenovirus 2 infection results in the inhibition of virus-specific RNA synthesis from all parts of the previously identified, very long (~28 kilobases) late transcriptional unit, except for the first ~400-800 nucleotides. It is suggested that DRB acts not as an inhibitor of RNA chain initiation, but rather causes 'premature' chain termination close to the promoter. A practical aspect of these findings may be that RNA sequences near promoter sites that are responsible for mRNA formation can be isolated from DRB-treated cells.

THE initiation sites for RNA synthesis (promoters) in adenovirus 2 (Ad 2) DNA during the infection of HeLa cells were the first promoters responsible for mRNA production to be identified in DNA-containing animal viruses or animal cells¹⁻⁴. As such, the DNA sequences in the region of the promoters, and the RNA transcribed from these regions are of considerable interest. A useful test for promoter proximity of nucleotide sequences in bacteria derives from the action of inhibitors like the rifamycins that allow chain completion but block chain initiation⁵. Thus, after rifamycin treatment, promoter proximal synthesis ceases first and promoter distal synthesis last. The nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was previously thought to act in eukaryotic cells by inhibiting transcriptional initiation of HnRNA, the possible precursor to mRNA⁶⁻⁹, and a test of this hypothesis was made possible based on the location of the promoter site for the majority of late adenovirus RNA synthesis. If DRB only stopped chain initiation, cessation of virus-specific nascent chain labelling would first occur near 16 (the major 'promoter region' late in Ad2 infection⁴), and then inhibition of labelling would gradually progress to the distal terminus of the transcribed region near 100 on the physical map. (Conventional description¹⁰ of the Ad2 genome places 0 at the left-hand end and 100 at the right-hand end of the DNA strand which is transcribed towards the right; each unit is 0.35 kilobases, 1% of the genome, the total length of Ad2 DNA

being 35 kilobases. Restriction endonuclease fragments of the DNA are designated by italics; for example, *Sma*II 11.6-18.2.) The results indicated that DRB did, in fact, inhibit Ad2 RNA synthesis near the origin of the transcription but, surprisingly, that RNA chains which possibly represent the first 400-800 nucleotides of the transcription unit continued to be labelled.

Late Ad2 RNA synthesis in DRB-treated cells

In a way similar to its effect on cellular HnRNA synthesis, DRB strongly inhibited both late Ad2 nuclear RNA synthesis (90-95%) and the appearance of label in Ad2-specific mRNA (>95%) (Table 1). Additional experiments demonstrated that a concentration of 75 μM DRB exerted maximal inhibition of Ad2 RNA synthesis (data not shown), as was also true for cellular mRNA synthesis^{11,12}.

The time course of inhibition of Ad2 RNA synthesis by DRB was then determined; an inhibition of chain elongation should inhibit incorporation immediately, whereas inhibition of chain initiation would inhibit incorporation more gradually because of the completion of already initiated chains. Ad2-infected HeLa cells were exposed to DRB for various periods and then labelled for 2 min with ³H-uridine. A pulse of this duration should be much shorter than the anticipated synthesis time

Table 1 Inhibition of late Ad2 RNA synthesis by DRB

	Nuclear RNA		Polysomal mRNA	
	c.p.m.	% of control	c.p.m.	% of control
Control	14,667	—	937	—
DRB	732	5.0	15	1.6

One sample of HeLa cells (5×10^7 per sample) infected with Ad2 was exposed to DRB (75 μM) 16 h after infection, and one served as control. After 30 min, 1 mCi of ³H-uridine (New England Nuclear, 26 Ci mmol⁻¹) was added to each sample and incubation continued for a further 35 min. Nuclear RNA and EDTA-released polysomal mRNA were prepared as described^{1,2,23}. Ad2-specific incorporation was measured by hybridisation to nitrocellulose filters containing 2 μg each of Ad2 DNA^{1,2}. Incorporation of ³H in the presence of DRB has been corrected for an inhibition of ³H-uridine transport into cells by a factor of 1.23 (ref. 11).

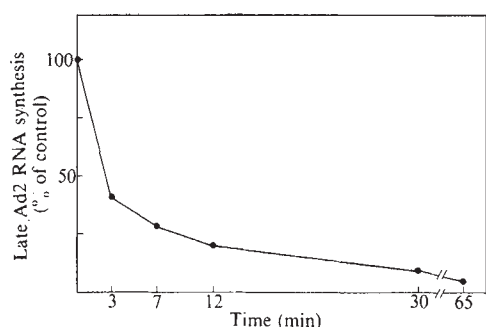


Fig. 1 Late Ad2 transcription in HeLa cells treated with DRB for various times. HeLa cells (1×10^8 cells per sample) were infected with Ad2 virus at a multiplicity of $\sim 2,000$ virus particles per cell. At 16 h after infection the cells were resuspended at 4×10^6 per ml and treated with DRB (75 μ M). At various times after treatment with DRB, 3 H-uridine was added to each culture (0.4 mCi per ml) and the cells labelled for 2 min. Nuclear RNA was extracted²³ and a one-tenth part hybridised to the nitrocellulose filters (three per sample) containing 2 μ g of Ad2 DNA. The figure presents the average of several independent experiments. The 65-min point represents data from the experiment in Table 1. 3 H incorporation in the presence of DRB was corrected for the inhibition of 3 H-uridine transport into cells (a factor of 1.23–2.5, depending on the duration of labelling¹¹).

(approximately 7–10 min) of the large late Ad2 transcript^{1,4,9}. Figure 1 shows that there was a gradual reduction in the amount of Ad2-specific nuclear RNA labelled in a 2-min pulse, with a near maximal effect by 15–30 min. This result is similar to the effect of DRB on cellular HnRNA synthesis in uninfected HeLa cells, except that Ad2 RNA labelling is inhibited by 90–95%, while cellular HnRNA labelling was inhibited by only 70% (ref. 11).

DRB acts close to the site of Ad2 RNA chain initiation

If DRB inhibits Ad2-specific RNA labelling by stopping chain initiation, labelling of RNA sequences complementary to DNA near the promoter will be the first to cease, and sequence labelling most distal to the promoter will persist the longest. HeLa cells, infected for 15 h with Ad2, were treated with DRB for 7 or 40 min and then pulsed with 3 H-uridine for 2 min. Nuclear RNA from these cells was hybridised to a series of restriction endonuclease fragments of the Ad2 genome. After the 7-min treatment, RNA complementary to DNA fragment 18–36 showed the most pronounced decrease, and there was less inhibition of labelled RNA specific for successive DNA fragments, progressing to the DNA fragment 89–100 (Fig. 2). After 40 min of DRB treatment the gradient of inhibition of nascent RNA labelling was still apparent, but inhibition was more extensive throughout the genome (Fig. 2) and by 60 min (not shown) transcription of the right-hand end of the genome was also inhibited by $> 90\%$. For example, in one experiment, the hybridisations to 89–93.5 and 93.5–100 were inhibited 93% and 95%, respectively. These results are consistent with the conclusion that DRB acts close to the site of RNA chain initiation.

The site of initiation of the large late Ad2 transcript has been pinpointed at between 16 and 17 on the genome⁶, so that the 11.6–18.2 fragment (*Sma*I-f) should hybridise approximately the first 600 800 nucleotides of the primary transcript. If DRB acted at the initiation site, the labelling of *Sma*I-f-specific RNA should be the first to cease in DRB-treated cells. This was not the case (Fig. 2); at least 20–25% as much labelled *Sma*I-f-specific RNA was formed in treated as in control cells.

In addition to the results with the *Sma*I-f DNA fragment, about 30–50% of the labelled nascent RNA that hybridised to the 3.0–11.1 (*Sma*I-e) fragment was resistant to DRB inhibition (Fig. 2). In addition to the many prominent late

Ad2 mRNAs that arise from sites to the right of 30 on the Ad2 physical map^{13–15}, there also exist mRNAs from the *Sma*I-e region that can be detected both by electron microscopy¹³ and pulse-labelling^{16,17}. Also, pulse-label experiments with *in vitro* nuclei² and with whole cells (Fig. 4) show that a second smaller transcription unit seems to function in this region. Thus, like the results with the large late transcription unit, DRB-resistant RNA was detected in the region of this small transcription unit.

The continued labelling of RNA in the presence of DRB could arise from: (1) a DRB-resistant leftwards-reading transcription unit beginning in *Sma*I-f; (2) a DRB-resistant rightwards-reading transcription unit beginning in *Sma*I-e and reading through *Sma*I-e and f but no further; (3) the site of action of DRB could be a short distance from the initiation site, rather than at the initiation site, thus allowing the continued (independently promoted) transcription of short RNA molecules from both DNA fragments 3–11 and 11.6–18.2. These possibilities can be tested by determining the strand-specificity of pulse-labelled RNA, and by determining the size of fragment-specific labelled RNA after treatment of cells with DRB.

Strand specificity of late Ad2 transcription

Pulse-labelled RNA obtained by labelling cells for 2 min at 16 h after infection was hybridised to separated strands of Ad2 DNA¹⁴. Over 95% of transcription occurred from the rightwards-reading strand (Table 2). Furthermore, the total RNA labelled in the presence of DRB remained $> 95\%$ specific for the rightwards-reading strand. In addition to separation of the strands of the total Ad2 DNA, strands of the *Sma*I-f fragment were separated¹⁵ and at least 90% of the *Sma*I-f

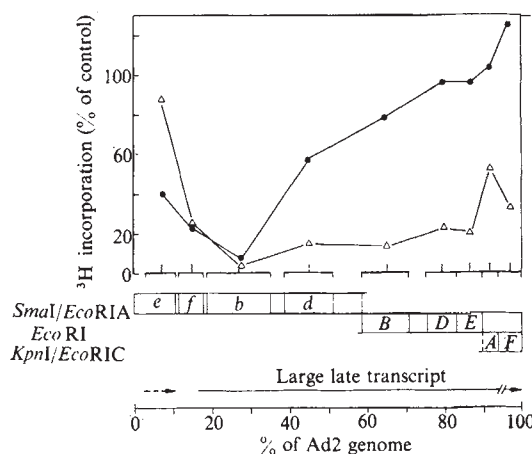


Fig. 2 Hybridisation across the Ad2 genome of 3 H-uridine pulse-labelled nuclear RNA late in infection after brief treatment with DRB. Ad2-infected HeLa cells were treated with DRB (75 μ M) for 7 (●) or 40 (△) min 16 h after infection, and pulse labelled with 3 H-uridine as described in Fig. 1. Nuclear RNA was extracted; the RNA was fragmented by treatment with alkali²⁴ to a length of ~ 200 –300 nucleotides, and hybridised to duplicate nitrocellulose filters (20 mm diameter) containing an amount of appropriate restriction fragments of Ad2 DNA equivalent to that contained in 2 μ g of total Ad2 DNA (2 μ g equivalent). RNA was dissolved in $2 \times$ TESS (NaCl 0.3 M, SDS 0.2%, EDTA 0.012 M, TES (N-Tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid; Sigma) 0.01 M, pH 7.4), and hybridisation was carried out in a total volume of 1 ml for 40 h at 68 °C. The total radioactivity hybridised was corrected for inhibition of 3 H-uridine transport by DRB (a factor of 1.8 (ref. 11)) and expressed as a % of the radioactivity hybridising in the control samples. The radioactivity (c.p.m.) hybridising to control filters was as follows: e, 1,519; f, 9,162; b, 11,531; d, 3,181; B, 3,785; D, 2,757; E, 1,468; K_A, 894; K_F, 682. These values correspond to the following molar ratios: 0.71, 5.28, 2.45, 1.00, 1.18, 1.40, 0.89, 0.89, 0.40, respectively. Map coordinates for DNA restriction fragments are taken from personal communications of C. Mulder.

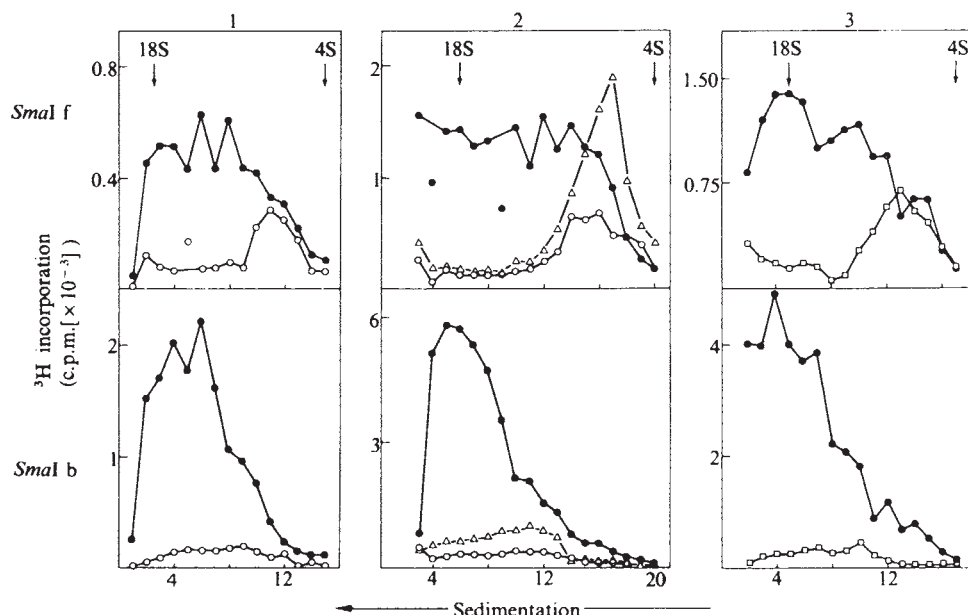


Fig. 3 Size of DRB-resistant *SmaIf*-specific late Ad2 RNA. Ad2-infected cells were exposed to DRB (75 μ M) for 7 (○), 40 (△) or 60 (□) min late in infection, and pulse-labelled with 3 H-uridine for 2 min (see Fig. 1). An untreated sample of infected cells served as control (●). The nuclear RNA was extracted and sedimented through a 15–30% sucrose gradient in 0.05 NETS in a SW40 rotor, such that 18S RNA sedimented close to the bottom of the gradient. RNA from each fraction $\geq$ 4S was broken with alkali and hybridised to duplicate 3-mm square nitrocellulose filters containing 1 μ g equivalent of Ad2 DNA restriction fragments *SmaIf* (11.6–18.2) and *SmaIb* (18.8–36.7) for 40 h at 68 °C in 2 $\times$ TESS. The RNA in experiments 2 and 3 was also hybridised to *SmaIc* (3.0–11.1) (Fig. 4). 3 H incorporation in the presence of DRB was corrected for the reduction in the transport of 3 H-uridine into cells (a factor of 1.6–1.9 (ref. 11)). Data presented in the three panels of this figure represent 3 separate experiments.

specific pulse-labelled RNA from both control and DRB-treated cells hybridised to the rightwards-reading strand (Table 2).

Size of DRB-resistant RNA

Pulse-labelled RNA from DRB-treated and control cultures was separated by sucrose gradient sedimentation to examine the sequence specificity of chains from $\sim$ 100 to $\sim$ 2,000 bases in length. The size-fractionated RNA was hybridised to the 11.6–18.2 (*SmaIf*) and the 18.8–36.7 (*SmaIb*) DNA fragments (Fig. 3). In the control culture, the size of the nascent RNA transcribed from *SmaIb* was almost all larger than about 1 kilobase (the maximum distance from the expected promoter site to the *SmaIb* regions) and much of the *SmaIb*-specific RNA sedimented to the bottom of the sucrose gradients. Very little *SmaIb*-specific RNA was labelled in the presence of DRB. The *SmaIf*-specific RNA that was labelled in the control culture (Fig. 3, upper panels) was both larger and smaller than about 1 kilobase, extending down to molecules as short as 100 nucleotides. These results are consistent with the conclusion⁴ that the promoter for the large rightward-reading transcript lies in *SmaIf*, but the 2-min pulse label allowed chains to be synthesised with labelled *SmaIf*-specific sequences which were as large as or larger than 1 kilobase. However, after treatment of cells with

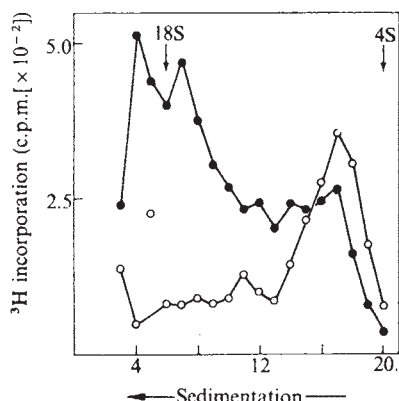
DRB, all of the labelled RNA that hybridised to *SmaIf* was $\sim$ 7–9S, the labelling of larger molecules containing *SmaIf* sequences being almost completely inhibited. In the 7–9S region, there were approximately equal amounts of labelled *SmaIf*-specific RNA in the control and drug-treated cultures.

In addition to the continued labelling of a short *SmaIf*-specific RNA in the presence of DRB, there was also continued labelling of short RNA chains complementary to the 3–11 (*SmaIc*) fragment (Fig. 4). Although the precise boundaries of a transcription unit in this region are not yet known, it is

Table 2 Strand specificity of late Ad2 transcription

	DNA on filter	Radioactivity hybridised	
		c.p.m.	c.p.m. % r+l
a Control	Total Ad2	6,908	—
	Ad2, r strand	4,189	98.8
	Ad2, l strand	50	1.2
DRB (7 min)	Total Ad2	816	—
	Ad2, r strand	933	98.2
	Ad2, l strand	17	1.8
b Control	<i>SmaIf</i> , r strand	909	92.8
	<i>SmaIf</i> , l strand	70	7.2
DRB (7 min)	<i>SmaIf</i> , r strand	196	90.7
	<i>SmaIf</i> , l strand	20	9.3
DRB (40 min)	<i>SmaIf</i> , r strand	211	88.3
	<i>SmaIf</i> , l strand	28	11.7

Fig. 4 Size of DRB-resistant *SmaIc*-specific late Ad2 RNA. Nuclear RNA of Ad2-infected cells, prepared as described in experiment 2, Fig. 3, was hybridised to duplicate 3-mm squares of nitrocellulose filters containing *SmaIc* (3.00–11.1) Ad2 DNA. ●, Control cells; ○, DRB treated cells.



For experiment *a*, nuclear RNA was prepared from DRB-treated (75 μ M) and untreated Ad2-infected HeLa cells pulse-labelled for 2 min with 3 H-uridine 16 h after infection (see Fig. 1). The total RNA input was $\sim$ 40,000 c.p.m. for the control cells and $\sim$ 20,000 c.p.m. for DRB-treated cells; duplicate filters containing 1 μ g of total Ad2 DNA or the equivalent rightward (r) or leftward (l) strands¹⁴ were used in separate hybridisation reactions. 32 P-labelled RNA prepared by *in vitro* transcription of total Ad2 DNA by *E. coli* RNA polymerase was included during the hybridisation as an internal control on the ability of r and l strands to bind RNA. The r/l ratio was 60:40. For experiment *b*, pooled RNA comprising 10% of each of fractions 3 to 20 of Fig. 3, experiment 2, were hybridised to DNA filters containing separated strands of *SmaIf*, 11.6–18.2 (prepared as described by Sharp *et al.*¹⁵). DNA was transferred directly from the gel slices to nitrocellulose filters using the technique of Southern *et al.*²². The ratio of hybridisation of 32 P-labelled transcripts of total Ad2 DNA synthesised *in vitro* by *E. coli* RNA polymerase to filters containing *SmaIf*, r strand and *SmaIf*, l strand was 45:55.

clear that one and perhaps two or three different short (1–1.5 kilobase) mRNA molecules complementary to the rightward strand of this region are labelled late in infection^{13,16,17}. Thus, the presence of a DRB-resistant, short, *SmaI*-specific RNA suggests that both the large and small late transcription units may be inhibited by DRB near to but not at the promoter site.

Discussion

These results demonstrate that DRB inhibits late Ad2 transcription by ~ 90% and mRNA formation by > 95%. Furthermore, it is quite clear that DRB acts at or close to the site of RNA chain initiation. The surprising observation is that short chains (7–9S) of RNA continue to be transcribed from the promoter proximal regions of the two late Ad2 transcription units even after extended exposure to DRB. Whether DRB acts by enhancing a physiological termination site about 400–800 nucleotides from the promoter or causes abnormal chain termination is unclear.

The main problem in proposing a specific mechanism of DRB action is that the details of Ad2 RNA chain termination, particularly any normal 'premature' chain terminations, are not yet known. In several prokaryotic transcription units, for example, the tryptophan operon on *Escherichia coli*^{18,19} and in λ bacteriophage²⁰, there exist RNA chain termination signals within 100–200 nucleotides of the chain initiation site, and short promoter-proximal RNA molecules have been identified in both these systems. In fact, transcriptional regulation in both these cases is effected by permitting or preventing synthesis past the site of 'premature' termination.

In Ad2-infected cells, there seems to be a termination site in the late, large transcription unit about 2 kilobases from its origin, in addition to a termination site somewhere near the end of the genome. An RNA peak of ~ 18S was detected by Weber *et al.*² in pulse-labelled RNA from isolated nuclei that hybridised mostly to DNA fragments 11.6–18.2 (*SmaI*f) and 18.8–36.7 (*SmaI*b). A 'short' RNA of similar size has been observed after 45-s pulse labels *in vivo* (R. Evans, M. Salditt-Georgieff and J.E.D., unpublished). However, the DRB-resistant RNA (Fig. 3) from the large late transcript that begins in *SmaI*f clearly does not proceed into *SmaI*b (Fig. 2). Thus the DRB-resistant RNA is not as long as the putative 'premature' termination product formed without the drug. Such a result, however, does not mean that DRB has nothing to do with a physiological termination signal. For example, a weak termination signal, 0.4–0.8 kilobases from the origin of RNA synthesis, that caused chain release only one-fifth of the time, would result in the normal accumulation of so little RNA in the 0.4–0.8-kilobase size class compared to the 2 kilobase

RNA that it would thus far have escaped attention.

This effect of DRB is also seen in other transcription units. A similar short RNA from the other active late transcription unit (3–11) is also labelled in the presence of DRB (Fig. 4). In addition, preliminary evidence shows that 'short' RNA is formed in the presence of DRB in all of the five Ad2 transcriptional units that are active early in infection, but again no mRNA is formed (P.B.S., N.W.F. and J.E.D., unpublished).

Thus, this drug would seem to be particularly useful in helping to label 'short' RNA chains that occur near the origin of transcription. In this connection, Tamm²¹ has observed that in isolated nuclei from DRB-treated HeLa cells, a 'short' (~ 7–9S) class of RNA continues to be labelled at a normal rate, whereas longer HnRNA molecules are inhibited by about 75%. In light of the Ad2 results, it is possible that these 'short' DRB-resistant chains transcribed from cellular DNA by RNA polymerase II²¹ are the promoter proximal regions of cellular transcriptional units.

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DNA lesions occur with loss of viability in embryos of ageing rye seed

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In dry seeds, fragmentation of nuclear DNA and activation of DNases occur in vivo during embryo senescence. This loss of DNA integrity could be the source of chromosomal aberrations and impaired transcription observed when seeds of low viability germinate.

A SEED which fails to germinate when given water and the appropriate environment for reactivation is said to have lost

viability¹. Seeds from low percentage viability stocks have a diminished ability to synthesise RNA and protein^{2–5}, show a failure of respiratory coupling⁶, loss of enzyme activities (dehydrogenases⁷, GTP-dependent transferase^{8–10}), impairment of ribosomes^{11,12} and the loss of long-lived poly A-rich RNA¹³; all are events associated with slow germination and loss of vigour. To date, however, little is known of the biochemical status of the genetic material during loss of viability.

In 1901, de Vries¹⁴ first questioned that a change in DNA might be the cause of poor germination and the high proportion of morphologically abnormal plants produced from old seeds of *Oenothera lamarckiana*. Later, high numbers of

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chromosomal aberrations were correlated with plants from low viability seed stocks¹⁵⁻¹⁸ and with plants produced from old seeds¹⁹. Chromosomal aberrations probably give a low estimate of the extent of genetic damage, for they are visible only when the cells divide at germination. It is clear, however, that damage to DNA must occur either *in vivo* during senescence of the embryo in the dry seed, or as an early event in germination. Biochemical studies are therefore necessary to determine overall damage to DNA and to verify the occurrence of lesions in the dry state.

With loss of viability in rye embryos, Roberts and Osborne²⁰ found no total loss of DNA per nucleus, but the amount of high molecular weight DNA which can be recovered by spooling (Marmur's method)²¹ is less in non-germinating embryos that had been non-viable (NV) for at least a year (3.1 mg DNA g⁻¹) than in those with 95% germination that were viable (V) (10.2 mg DNA g⁻¹). However, whether this reflected breakage of DNA *in vivo*, or was the result of extraction procedures, was not determined. Damage to DNA

has been correlated with cellular senescence in a variety of animal tissues²²⁻²⁵, but in these studies also, the possible degradation of DNA during extraction was not excluded.

Although the ability to synthesise both RNA and protein at imbibition is progressively impaired with loss of viability, embryos that have just reached non-viability (0% V) synthesise some RNA, but no protein^{2,3,5,13,26}. Lesions in DNA could contribute to such reduced template activity, and possibly also to a faulty transcription. In comparing the integrity of DNA in V and NV embryos by several methods and in the absence of detectable enzymic degradation during experimental procedures, we have found and present here evidence for the presence of breaks in the DNA of dry NV embryos *in vivo* and for the activation of deoxyribonucleases with loss of viability.

Loss of integrity of DNA *in vivo* in NV embryos

Experiments were carried out on embryos separated from dry grains of rye by the mass method of Johnston and Stern²⁷. DNA was isolated from V and NV embryos, and the sedimentation profiles compared in neutral and alkaline sucrose density gradients. Centrifugation of a nucleic acid extract from V and NV embryos in a neutral sucrose density gradient to give the sedimentation profile of native DNA is shown in Fig. 1. Duplex DNA from NV embryos consisted of a more homogeneous population of lower mean molecular weight molecules than similarly extracted DNA from V embryos. Although this suggests that NV DNA could be cleaved in the dry embryo during loss of viability, such experiments do not exclude the possibility that the difference between V and NV DNA resulted from differential fragmentation of NV DNA during preparation procedures, or by nucleases released on cell disruption. DNA molecules are, by virtue of their large size, very susceptible to shear even by the most gentle handling procedures. DNA prepared by methods involving deproteinisation will not therefore be in the *in vivo* high molecular weight form. This difficulty can theoretically be overcome by isolating whole cells and lysing them on the top of an alkaline sucrose density gradient²⁸. The DNA is thereby released from the cell and is denatured directly within the gradient with minimal shear. In plants, direct cell lysis is not possible because of the presence of the cell wall, but analyses of DNA can be appropriately carried out by first isolating nuclei and then lysing these on a gradient.

Nuclei prepared separately from V and NV embryos and from a mixed embryo sample of an equal number of V and NV embryos were lysed on top of alkaline sucrose density gradients and the denatured DNA subjected to centrifugation as described in the legend for Fig. 2. Two different buffers were compared for the nuclear preparations. Buffer A (ref. 29), which has been used successfully for rat liver, contained polyamines as stabilising agents (replacing divalent cations, for example, Mg²⁺ or Ca²⁺) to minimise any DNase activity during extraction. This was preferable to a buffer containing 5 mM Mg²⁺ which has been used for isolating nuclei from wheat embryos (A. J. Trewavas, personal communication). A typical experiment with buffer A is shown in Fig. 2.

In a series of experiments, comparison of the sedimentation profiles of single-stranded V and NV DNA consistently revealed a lower mean molecular weight of NV DNA, regardless of which buffer was used to prepare the nuclei. Some high molecular weight DNA was, however, always present in NV embryos, as seen in Fig. 2. The sedimentation profile of DNA from nuclei prepared from the mixed V and NV sample clearly shows the individual V and NV peaks. The amounts of DNA in the two peaks in the V and NV gradient are roughly 50% of those in the separate V and NV gradients, sedimenting in the same individual positions. As the degree of mechanical shear is identical in the three samples, the profiles obtained suggest that no enzymic degradation of DNA had occurred during the extraction procedures. Therefore, the results are

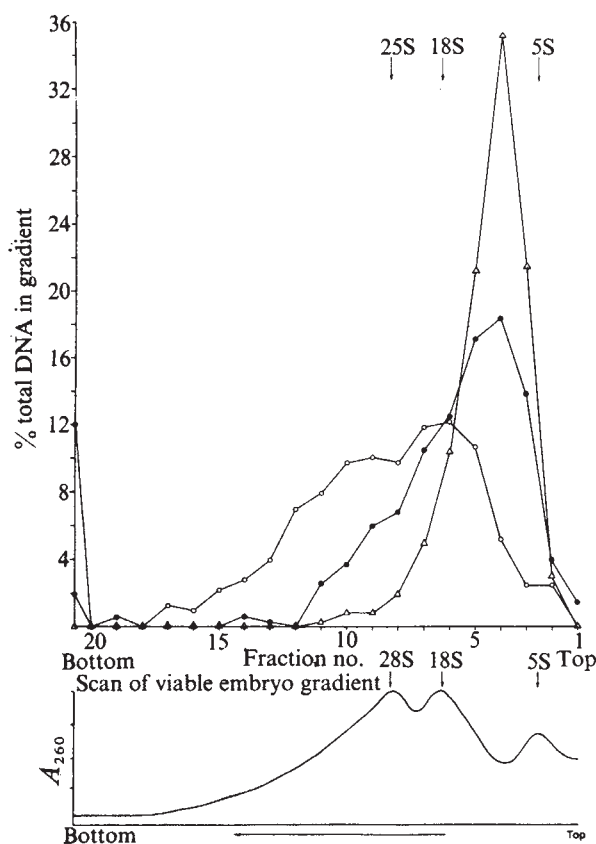


Fig. 1 Fractionation of DNA from V and NV embryos in 5–20% neutral sucrose density gradients. Rye embryos (600 V or NV) were homogenised in 1.5 ml 0.15 M NaCl in 0.1 M EDTA (pH 8.0) and SDS (sodium dodecyl sulphate) added to 2.5%. The homogenate was heated for 10 min at 60°C and NaClO₄ added to 0.5 M. Samples were deproteinised by shaking with an equal volume of chloroform: isoamyl alcohol (24 : 1) followed by three successive extractions with equal volumes of phenol : chloroform (1 : 1). The extracted aqueous phase was dialysed overnight against 1,000 volumes of a solution of 0.15 M lithium acetate (LiAc) in 2% SDS at 2°C. An aliquot of DNA from V embryos was sheared by sonication at 22 kc s⁻¹ for 5 min in an MSE 150 W ultrasonic disintegrator. The DNA samples were layered over a 5–20% sucrose gradient in LiAc–SDS solution and centrifuged in a Beckman SW25 rotor at 25,000 r.p.m. for 6.5 h at 10°C. Fractions (1.2 ml) were collected from the top of the gradient, 100 µg bovine serum albumin added as carrier, and perchloric acid (PCA) added to 10%. The deoxypentose insoluble in 10% PCA was determined in each fraction by the modified diphenylamine reaction²⁹. Total DNA loaded onto gradients was: V, 750 µg (○); V (sonicated), 750 µg (Δ); NV, 700 µg (●).

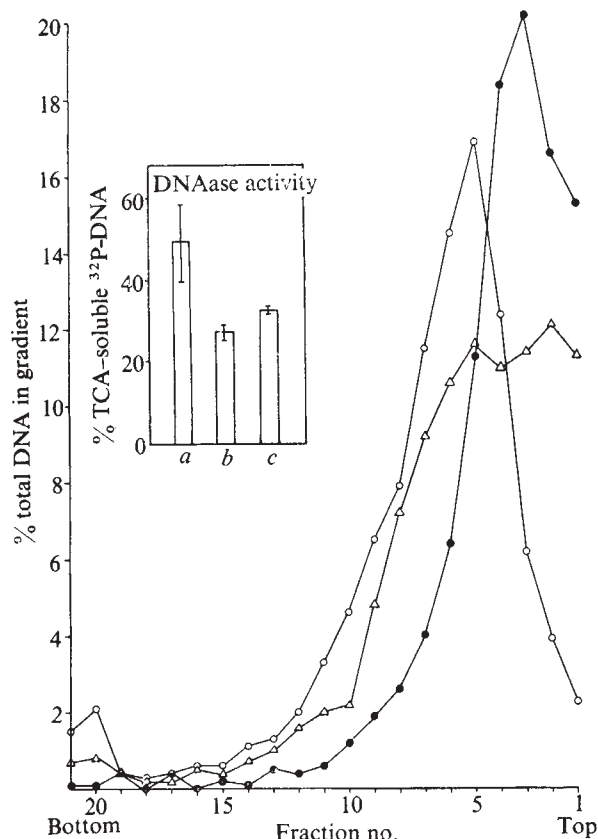


Fig. 2 Fractionation of DNA from isolated nuclei of V and NV embryos on 5–20% alkaline sucrose gradients. Nuclei from 800 V or NV embryos were prepared by a modification of the method of A. J. Trewavas (personal communication). All procedures were carried out at 0–2 °C. Preparations of the V(○), NV(●) and V+NV(△) nuclei were carried out concurrently. Hand-selected rye embryos were homogenised in 1.5 ml of buffer A²⁹ consisting of 0.3 M sucrose in 10 mM KCl, 15 mM NaCl, 15 mM β-mercaptoethanol, 15 mM Tris-HCl (pH 7.4 at 20 °C), 0.15 mM spermine and 0.5 mM of spermidine. The homogenate was diluted to 10 ml and filtered through three layers of Miracloth, the residue washed with 28.0 ml of the same sucrose-buffer. Triton X-100 was added to 1% and nuclei were pelleted at 625g for 6 min. This crude nuclear preparation was resuspended in 0.2 ml of buffer A and pipetted into a lysing layer consisting of 0.8 ml of 0.4 M NaCl, 10 mM EDTA and 0.15 M NaOH (pH 13.0) overlaid on top of a 5–20% alkaline sucrose gradient in 0.3 M NaOH, 0.7 M NaCl, 10 mM EDTA (pH 13.0) (ref. 54). The lysate was incubated in the dark at room temperature for at least 2 h to allow time for complete denaturation of DNA. The gradients containing V, NV and V+NV DNA were then centrifuged simultaneously in a Beckman SW25 rotor at 12,000 r.p.m., for 14.5 h at 10 °C and 1.2 ml fractions collected. The DNA in each fraction was determined as described in the legend to Fig. 1. Inset, nuclei isolated in buffer A. Test for DNase activity during the preparation of nuclei. DNA spooled²¹ from log phase *E. coli* strain B grown in the presence of 1 mCi ³²P₄. The spooled ³²P-DNA sample was dissolved in and dialysed against 0.1 × SSC (15 mM NaCl, 1.5 mM tri-sodium citrate) and purified by RNase A (EC 2.7.7.16) treatment (20 μg ml⁻¹ in 1.0 × SSC, pH 5.0, preheated at 90 °C for 10 min to destroy DNase activity) for 2.0 h at 33 °C followed by incubation for 4.0 h with 200 μg ml⁻¹ pronase B (predigested, 1.5 h at 33 °C) in TKM buffer (50 mM Tris-HCl, 50 mM KCl, 10 mM MgCl₂, 5.0 mM β-mercaptoethanol and 10 μg ml⁻¹ chloramphenicol) pH 7.0. The sample was deproteinised 3 times with phenol:chloroform (1:1), the purified ³²P-DNA spooled from the aqueous layer, and dialysed overnight at 2 °C against 1,000 volumes 0.1 × SSC. The ³²P-DNA was purified by this method twice. The final dialysis was against 1,000 volumes of buffer A. Aliquots of ³²P-DNA were incubated for 1.0 h at 2 °C with equal volumes of a, extraction buffer (buffer A), b, 625g nuclear pellet from NV embryos resuspended in buffer A, c, 625g supernatant from the NV nuclear preparation. The reaction was stopped with 10% PCA, and the acid-soluble material assayed for radioactivity by Cerenkov counting as a measure of the ³²P-DNA that had been degraded by DNase activity. The results are representative of six different experiments.

taken to illustrate the higher proportion of smaller molecular weight DNA from NV compared with V embryos.

As we describe later, there is a significant level of nuclease activity in NV embryos. To determine whether these enzymes could function during the preparation of nuclei, samples from each stage of preparation were tested for DNase activity by using a sensitive radioassay. For this, ³²P-labelled DNA was prepared by spooling²¹ from *Escherichia coli* grown in medium containing ³²P₄. The ³²P-DNA was incubated in identical conditions of time and temperature with each fraction (nuclear pellet and supernatant) obtained during the preparation of NV nuclei. No significant difference in DNA degradation; as judged by increase in TCA-soluble radioactivity, was detected between the buffer control and different sample extracts (Fig. 2 inset) at any time.

Additional evidence that breaks occur *in vivo* in nuclear DNA during loss of viability has been obtained from tests for *in situ* single-stranded 3'-OH tails using the enzyme calf thymus 3'-terminal deoxynucleotidyl transferase (EC 2.7.7.31). When median sections from individual embryos (fixed and prepared for autoradiography) are incubated with the enzyme and ³H-dCTP, dCMP is added specifically to any free 3'-OH termini to form poly dC-tails³⁰. The extent of this addition in deproteinised (+HCl) and untreated sections (–HCl) with the appropriate background controls, has been determined in V embryos (95% germination) and in embryos which during storage have just lost viability (0% V) but are still capable of synthesising RNA though not protein³. The frequency and intensity of silver graining over nuclei from the different treatments are shown in Table 1. The proportion of nuclei showing incorporation (hence the presence of single-stranded DNA tails)³⁰ was significantly higher in 0% V than in V embryos. In addition, a higher total level of incorporation was observed on 0% V nuclei. As this technique excludes DNA degradation due to extraction procedures and tests for DNA breaks in fixed embryo sections, the results indicate a larger number of single-stranded breaks in the DNA of 0% V embryos.

From the evidence for a lower mean DNA molecular weight in NV embryos, and the increased incidence of *in situ* single-stranded breaks in 0% V embryos, we conclude that *in vivo*

Table 1 Incorporation of ³H-dCTP into median 3-μm sections cut from fixed and wax-embedded preparations of V and 0% V embryos

Pretreatment	Mean no. of grains per nucleus (above background)	% Nuclei showing incorporation > 4 grains	% Nuclei with > 10 grains	% Nuclei with > 20 grains
0%V	6.4 ± 0.2	53.0 ± 3.5	17.3 ± 0.3	3.3 ± 0.3
HCl	7.1 ± 1.3	67.5 ± 3.0	21.8 ± 1.0	2.8 ± 2
V	4.2 ± 0.1	34.0 ± 5.6	6.4 ± 0.2	0
HCl	4.0 ± 0.1	40.3 ± 1.9	3.8 ± 2.0	0
V and 0%V + DNAase	0	—	—	—

The embryos were incubated on glass slides with calf thymus 3'-OH terminal deoxynucleotidyl transferase (EC 2.7.7.31). Sections were incubated for 3 h at 37 °C with five units of enzyme in 0.2 M sodium cacodylate (pH 7.5), 1 mM CoCl₂, 1 mM β-mercaptoethanol and 5 μl ml⁻¹ 1% Triton X-100 with 10 μCi ml⁻¹ ³H-dCTP. Some sections were pre-treated for 30 min at 20 °C with 10 mM HCl to deproteinise the chromatin, rinsed and dried before incubation with precursor and enzyme. Sections were well rinsed in 150 mM NaCl followed by 5% TCA (containing 1% sodium pyrophosphate), then water, and finally dehydrated to 80% ethanol and air dried, before coating with Kodak AR10 stripping film for preparation of the autoradiographs. Method of Modak and Bollum³⁰. Grain counts were made over 200 nuclei from each embryo in each treatment. Values are ± range of duplicate embryo samples. Background = 4 grains per nucleus. Background levels of graining were determined by: excluding enzyme; treatment of sections with DNase (100 μg ml⁻¹ for 30 min at 37 °C) either before or after incorporation with enzyme; mean number of grains over equivalent areas of cytoplasm outside nuclei and of adjoining emulsion.

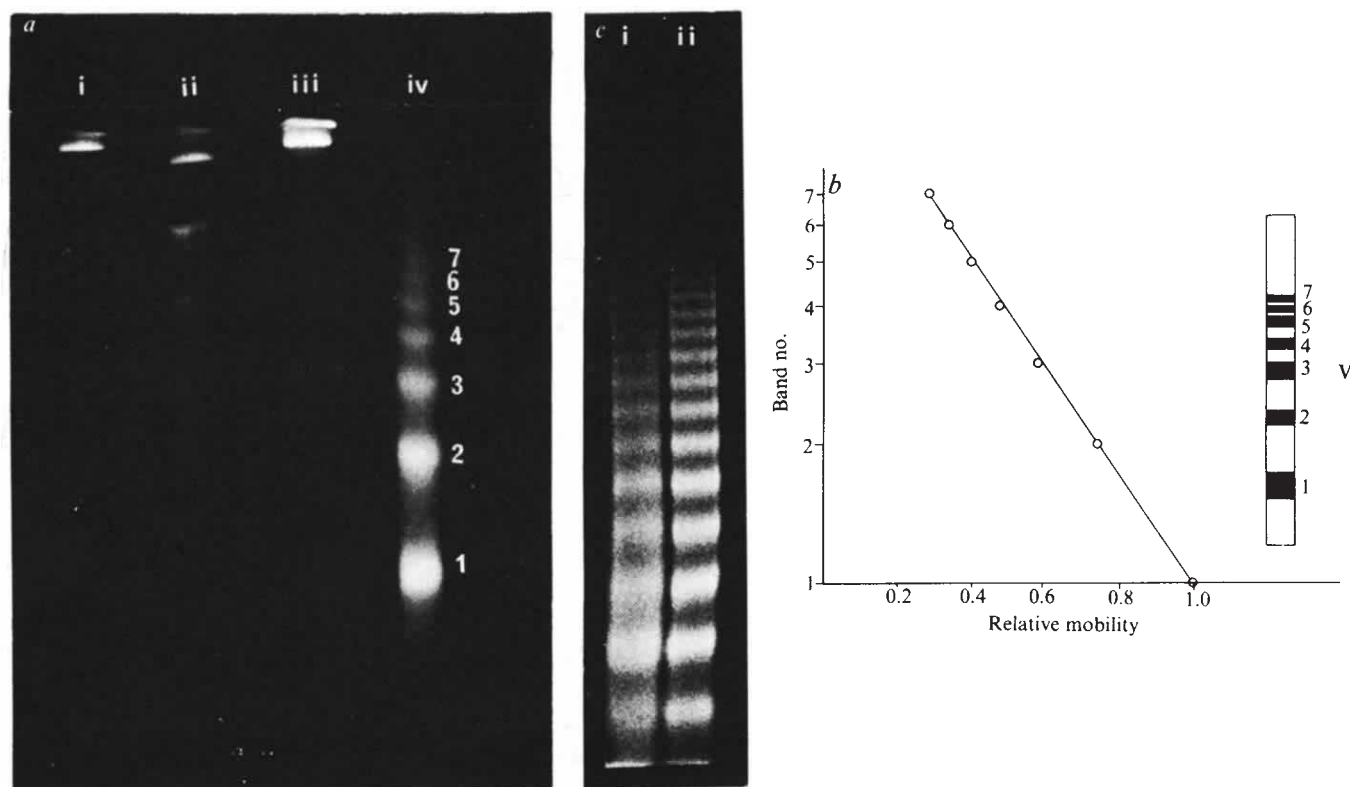


Fig. 3 Polyacrylamide gel electrophoresis of DNA fragments produced by micrococcal nuclease digestion of purified V and NV nuclei. Crude nuclear preparations were obtained from 600 V and NV embryos as described in the legend to Fig. 2, and purified as follows: the 625g pellet was resuspended in 1.0 ml 40% metrizamide (in 0.3 M sucrose-buffer A), and layered onto a two-step gradient consisting of 40% and 50% metrizamide in sucrose-buffer A. The gradients were centrifuged in a Beckman SW65 rotor at 25,000 r.p.m. for 15 min at 2 °C. The nuclear layer formed at the 40–50% metrizamide interphase was removed and the nuclei repelleted at 625g. (Nuclei were free of cytoplasmic RNA and cell wall contamination as judged by electron and fluorescence light microscopy.) Preparation of micrococcal nuclease digestion products: V or NV nuclei were resuspended in sucrose-buffer A and 0.8 ml incubated for 30 s at 37 °C with 10 units micrococcal nuclease (EC 3.1.4.7.) in 1.0 ml sucrose-buffer A containing 1.0 mM CaCl₂. The reaction was stopped by the addition of SDS to 2.5%, and an equal volume 0.3 M NaCl and 0.2 M EDTA (pH 8.0) and incubation at 60 °C for 10 min. The DNA was then deproteinised as described in the legend to Fig. 1. The aqueous phase was dialysed against 1,000 volumes electrophoresis buffer (40 mM Tris-acetate, pH 7.8, 20 mM NaAc, 20 mM EDTA) at 2 °C. The DNA samples were concentrated to 0.1 ml by surrounding the dialysis bag with Carbowax 20,000, and fractionated by electrophoresis on either polyacrylamide disc gels⁵⁵ or agarose slab gels at room temperature. Approximately 10–15 µg of DNA was loaded in each sample slot. Gels were stained for 10 min in 20 µg ml⁻¹ ethidium bromide (in 1.0 mM EDTA, pH 7.0), destained in 1.0 mM EDTA, pH 7.0, and photographed under ultraviolet light. *a*, DNA samples from micrococcal nuclease digests of V nuclei fractionated on 2.5% polyacrylamide disc gels for 2.0 h at 5 mA per tube. (i) V nuclei lysed at 0 °C with reaction termination mixture as above; (ii) as (a) with added 25S and 18S rRNA from rye embryos as markers; (iii) V nuclei incubated with 0.1 ml 10 mM CaCl₂ for 30 s at 37 °C; (iv) V nuclei incubated with 10 units micrococcal nuclease 30 s at 37 °C. *b*, semilogarithmic plots of DNA band number (products of micrococcal nuclease digestion) versus mobility relative to the smallest size class of fragments. *c*, Micrococcal nuclease digests of V and NV nuclei. Nuclei isolated from V and NV embryos were incubated with 2 units ml⁻¹ micrococcal nuclease at 0 °C for 10 min. Digests were then incubated with 100 µg ml⁻¹ RNAase A for 10 min at 37 °C, and the DNA extracted and fractionated on 2% agarose slab gels at 35 V and 25 mA for 16 h. DNA (10 µg) from: (i) NV nuclei; (ii) V nuclei.

breaks do occur in nuclear DNA in the dry state during loss of viability.

The nature of the DNA lesions

Because loss of viability is faster at higher temperatures and higher humidities, stochastic mechanisms alone are precluded, and a nuclease action is the most likely way for DNA to be reduced to a lower molecular weight. Indeed, a higher DNase activity has been shown to be associated with the spooled DNA and supernatant fraction of NV compared with V embryos²⁰. Although both endo- and exonucleases could be involved, exonuclease degradation *in vivo* must be slight, for no total loss of DNA occurs with loss of viability²⁰.

Recent advances in our understanding of the primary organisation of chromatin and methods employing endonucleases to probe this substructure have facilitated our investigation of the kind of nuclease action responsible for DNA lesions in NV embryos. The current model of chromatin substructure³¹ envisages repeating nucleoprotein subunits of

DNA and histone (nucleosomes). Isolated nuclei of rat liver when incubated with added endonuclease *in vitro*, produce a series of DNA fragments consisting of multiples of 200 base-pairs³², as demonstrated by polyacrylamide gel electrophoresis. Additionally, the action of endogenous endonucleases *in vivo* can lead to breakdown of DNA within the tissue, as shown by the separation of multiples of DNA fragments of discrete molecular weight classes from degenerating embryonic mouse liver cells³³. The presence of such endonucleases has been tested in rye embryo by assaying for repeating size fragments in the low molecular weight NV DNA. Such tests first required the demonstration of a repeating subunit structure in rye chromatin.

We have established (Fig. 3*a* and *b*) that rye contains an organised nucleosome structure, and that this substructure is not lost as embryos become non-viable (Fig. 3*c*). As Fig. 3*a* shows, when nuclei of V rye embryos are incubated with micrococcal nuclease, a series of DNA fragments are generated. The straight-line relationship obtained from a semi-logarithmic

plot of band number against mobility (relative to the smallest size class of fragments) demonstrates (Fig. 3b) that the DNA bands are integral multiples of the smallest DNA size class. Comparisons of micrococcal nuclease digests which were subjected to extensive RNase A digestion before electrophoresis show (Fig. 3c) that the nucleosome nature of rye chromatin is retained overall after loss of viability, and the pattern is not attributable to any contaminating RNA fragments.

Comparison of the molecular weight distribution of DNA from nuclei of NV and V embryos shows that the majority of the DNA of both NV (Fig. 4a(i) and Fig. 4b(i)) and V (Fig. 4a(ii)) nuclei migrates a short distance only into the gel and consists of two bands. In NV nuclei, however, an additional diffuse component is present that is not RNA (Fig. 4a(iv) and Fig. 4b(ii), (iii) and (iv)) and is of higher molecular weight than the nucleosome monomer (Fig. 4a(ii)), which migrates throughout the middle of the gel. [A description of the determination of the rye nucleosomal DNA repeat (200 base-pairs with a 140 base-pair core) is described elsewhere³¹.] Such a spread could result from either single-stranded or double-stranded breaks in the chromatin DNA. If single-stranded breaks occur, and there is evidence that some are present (Table 1), they do not occur as discrete multiples of a size class when separated on denaturing gels (Fig. 5(i)), nor are specific fragment classes generated in NV embryos that have been imbibed for 18 h (Fig. 5(ii)). However, if only a few fragments of each size class were generated by an endonuclease activity, as described by Williamson³³ or Hewish and Burgoyne³⁵, the pattern could well be masked by a concomitant exonuclease activity. Alternatively, endonucleases with differing specificities and rates of reaction could yield heterogeneous products³⁶. It is also known that micrococcal nuclease digestion of chromatin in which the proper histone-DNA association in the nucleosome core is disturbed does not yield the characteristic band pattern in DNA, but rather a population of heterogeneous molecular weight fragments³⁷. Therefore, even though nucleosomes are still present in the nuclei of NV embryos³⁴ (Fig. 3c), the possibility cannot be discounted that the histone-DNA complex in NV embryos may not be intact overall, hence the irregular fragmentation pattern.

The cause of DNA lesions

The analysis of NV DNA by gel electrophoresis indicates the following possibilities for DNA degradation: (1) the action of exonucleases alone, (2) a combination of endo- and exonucleases, and (3) the action of endonucleases alone, perhaps on regions of chromatin in which the nucleosome structure is disrupted.

Tests for nucleases in rye embryos from a number of different stocks have shown that the activity of DNase in post-ribosomal supernatants from equal numbers of embryos is higher in NV than in V despite their lower content of soluble protein extracted. In the absence of evidence for protein synthesis in these dry embryos, the difference must result either from some form of activation of pre-existing latent enzyme or from a preferential stability or extractability of nucleases from NV embryos. Any preincubation of the V and NV supernatants before addition to the DNA substrate does not enhance the extent of DNA degradation (Fig. 6), so that an enzymic activation of DNases in the NV supernatants seems unlikely³⁸. Activation of preformed DNase molecules during dry storage is therefore an alternative. For the two samples of embryos specifically studied, the activity of DNase in NV supernatants is reduced by approximately 50% by the addition of supernatants from a similar number of V embryos (Fig. 6), and over the range of protein concentrations studied, supernatants from other stocks of viable embryos also reduce the nuclease activity of NV supernatants in mixing experiments. Total inhibition of NV DNase activity is, however, never achieved, and the inhibitory property of V supernatants is destroyed by heating (Fig. 6).

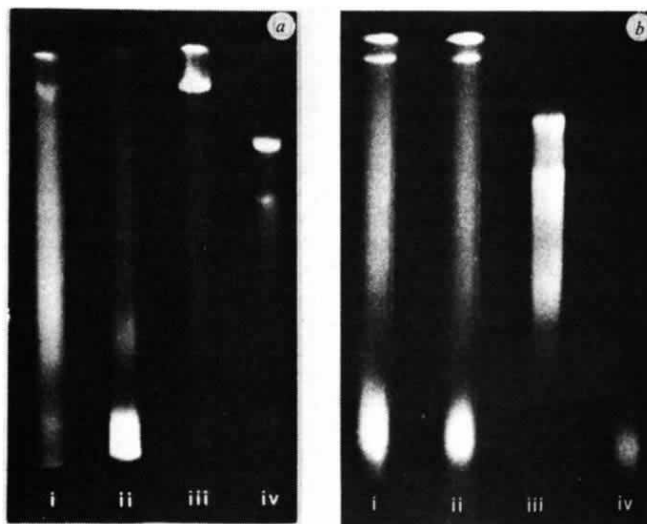


Fig. 4 Separation of high molecular weight DNA isolated from nuclei of V and NV embryos. Purified nuclei were prepared in buffer A from 800 V and NV embryos, and the DNA purified as described in Fig. 3. Electrophoresis on 2.5% polyacrylamide disc gels at 5 mA per tube for 2 h at room temperature was as described in Fig. 3. *a*, Fractionation of DNA from (i) NV nuclei; (ii) V nuclei incubated with 10 units micrococcal nuclease for 6 min at 37 °C (to visualise nucleosome monomer); (iii) V nuclei; (iv) rye embryo rRNA (25S and 18S). *b*, Fractionation of DNA from NV nuclei and test for RNA content; all samples incubated in buffer A for 1.0 h at 2 °C. (i) NV nuclei; (ii) NV nuclei incubated with 100 $\mu\text{g ml}^{-1}$ RNase A (EC 2.7.7.16) (preheated in buffer A at 90 °C for 10 min to inactivate DNase); (iii) rye embryo rRNA (25S and 18S); (iv) rye embryo rRNA + 100 $\mu\text{g ml}^{-1}$ RNase A (preheated in buffer A as in (ii)).

Fractionations on G25 Sephadex have indicated that the low DNase activity of V supernatants cannot be attributed to the presence of a low molecular weight inhibitor absent from the NV supernatants³⁸.

The cellular location of the active DNases in rye remains unknown. In yeast, DNase and an inhibitor are present in the nuclear fraction³⁹. In mammalian cells endonucleases cleave DNA molecules in dying cells and may also be associated with the nucleus⁴⁰. As the DNA of rye embryos is cleaved in the dry state (10% moisture content), it is likely that here too nuclease activity is located within the nucleus. However, because high molecular weight DNA can be isolated from nuclei of NV embryos even after they have been imbibed for 18 h (Fig. 5), such nucleases must have a slow rate of activity *in vivo*. Predictably, the accumulation of breaks in the embryos of dry seeds would also be a slow and gradual process, in contrast to that observed in hydrated cells⁴⁰.

Role of DNA lesions in loss of viability

Loss of viability in seeds is accompanied by a progressive diminution in the capacity for protein synthesis at imbibition. Although this can, in part, be attributed to failures in the protein synthesising systems^{8,11}, it is also associated with a decline in new RNA synthesis in all cells of the embryo^{3,4,26}. The RNA synthesised by the just 0% V embryos is nuclear in origin^{3,26}, is all of low molecular weight^{5,13}, and may represent incomplete or faulty RNA transcripts from an impaired template, and include nonfunctional messages. Unequivocal evidence for the accumulation of DNA breaks in embryos at specific intermediate stages during loss of viability is difficult to obtain. In a 50% viable stock, for example, 50 embryos of every 100 will be NV, the remainder are V but of low vigour. Unless the embryos are tested for germination, there is no known feasible method for distinguishing V from the NV. For seeds in the dry state, therefore, it is not possible to separate and assay the partially impaired, low performance

V embryos alone. (The declining rate of protein synthesis with loss of viability is not, however, simply proportional to the numbers of NV embryos within the sample but reflects an impaired embryo 'vigour' in all the seeds^{11,41}.) In NV embryos (Fig. 4a(i) and b(i)), a proportion of the DNA is degraded from a length of 3,000 base-pairs to molecules as small as 200 base-pairs (estimated by comparing the smallest NV DNA fragment with V DNA nucleosome monomers). Some breaks could be presumed to occur before the embryos become 0% viable (Table 1), and they could contribute to the progressive failure of RNA synthesis at germination. They could also account for the increased incidence of chromosomal aberrations with loss of viability¹⁵⁻¹⁸.

The number of breaks that must be incurred in the DNA template before lethality varies widely between different organisms. In *E. coli*⁴² and in yeast⁴³ a single double-strand break may be a lethal event, but more than 100 may be required for lethality in the ovaries of Chinese hamster⁴⁴. Double- rather than single-stranded breaks lead to loss of priming activity following DNase action on native calf thymus DNA, and the length of the DNA fragment produced is critical⁴⁵, reduction of the DNA from 9,000 base-pairs to approximately 3,000 resulting in a 40% decrease in the priming

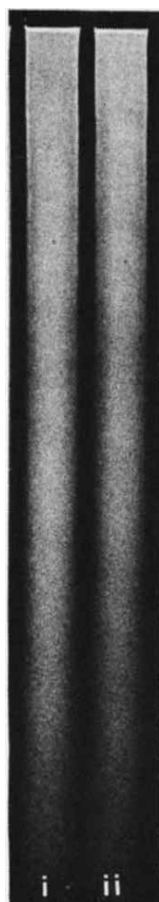


Fig. 5 Fractionation of NV DNA under denaturing conditions. Crude nuclear preparations as described for Fig. 2 were obtained from (i) 400 NV embryos which had been imbibed in H₂O (containing 10 µg ml⁻¹ chloramphenicol) for 18 h at 25 °C; (ii) 400 dry (unimbibed) NV embryos. The nuclei were resuspended in 1.0 ml preheated RNase A (100 µg ml⁻¹ in buffer A) and incubated at 2 °C for 1.0 h as described in Fig. 4. DNA was extracted as described in Fig. 3 and denatured by boiling in 50% deionised formamide for 10 min. The denatured DNA samples (about 20 µg) were fractionated under denaturing conditions in 4% polyacrylamide slab gels containing 7 M urea (in electrophoresis buffer) at 25 mA and 35 V for 16 h at room temperature. DNA from V embryos does not enter the gel and hence is not shown.

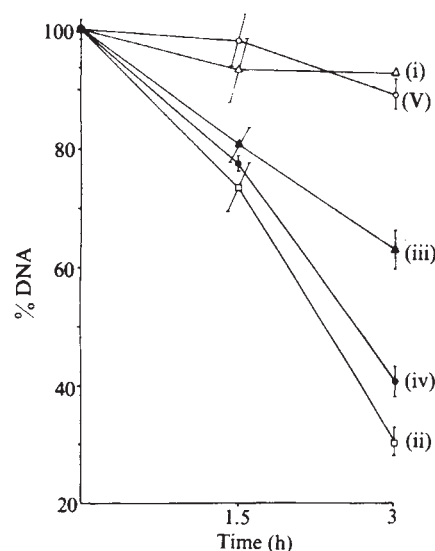


Fig. 6 DNase activity in extracts of V and NV embryos. V or NV (480 of each) was homogenised in TKM buffer, pH 6.0, including 10 µg ml⁻¹ chloramphenicol. Homogenates were centrifuged at 38,000g for 20 min at 2 °C and the supernatants made up to 10.5 ml. DNase activity in V and NV supernatants was determined by incubation of 0.5 ml aliquots with an equal volume of calf thymus DNA (100 µg ml⁻¹) in TKM buffer, and determining the PCA-insoluble DNA after 0, 1.5 and 3 h at 30 °C. The reaction was stopped by cooling the samples in ice and adding PCA to 10%. The precipitates collected after 24 h at 0 °C were then washed twice in ethanol and ethanol: ether (3:1), drained and resuspended in 1.0 ml 10% PCA and heated at 90 °C for 7 min. Deoxypentose was estimated by the modified diphenylamine reaction⁵³ and soluble protein by the method of Lowry⁵⁶. V (i)(△) and NV (ii)(□) supernatants were diluted with an equal volume of TKM buffer to give solutions of 130 and 45 µg ml⁻¹ protein, respectively, and assayed separately with the calf thymus DNA substrate; combined DNAase activity of V and NV supernatants was tested by mixing equal volumes of the V with the NV (iii)(▲). Effects of denatured V and NV supernatants were tested by heating them at 60 °C for 15 min before addition to equal volumes of the non-denatured NV (iv)(●) and V (v)(○) supernatants and calf thymus DNA substrate. To test for DNase activation by protease or other enzymic modification, samples of V and NV supernatants were preincubated at 30 °C for 0.5 h before addition of calf thymus DNA. Results (not shown) were within the range of (i) and (v) and (ii) and (iv), respectively³⁸. Bars=range of duplicates. V (i) at 3 h, single value.

capacity. By comparison with this degree of DNA breakage, the small fragments observed in NV embryos (about 200 base-pairs (Fig. 4)) could well account for the failure in RNA synthesis with loss of viability^{3,13,26}. The presence of single-stranded breaks in DNA does not, however, necessarily imply impaired cellular function, for a period of increasing single-stranded lesions has been observed during the active development of sea urchin embryos^{46,47}.

Both single- and double-stranded breaks in DNA are readily repaired in actively growing cells of bacteria and mammals⁴⁸. In plants, chromosomal aberrations are reported to decline in number following progressive cell cycles in the root tips of germinating onions⁴⁹, and repair processes have been indicated in carrot cell cultures⁵⁰. Seeds maintained in a fully imbibed but dormant state show a low incidence of chromosomal aberrations compared with those stored dry, and maintain a high capacity for germination, suggesting that a DNA repair process in imbibed embryos is possible^{18,51}. For example, dormant lettuce seeds given periods of imbibition between γ-irradiation treatments, show a lower incidence of radiation-induced chromosomal aberrations on germination⁵². Repair seems unlikely during dry storage of seeds, and an accumulation of breaks in DNA could eventually reach a level too high to be repaired at germination. Furthermore, if

the enzymes of a DNA repair system are representative of the more labile protein components of the cell^{7,8}, template restoration in embryos of low viability would then be defective or lacking. Indeed, the onset of DNA replication before cell division is known to be appreciably retarded in low viability material²⁶.

Using evidence from the several approaches presented here, we propose that a loss of integrity of genetic material can occur in the embryos of seeds during loss of viability in the dry state. Breaks in DNA, coupled with failures in the repair capability could cause impaired coordination of synthetic processes, leading to a cellular senescence and the eventual inability of a seed to germinate.

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letters to nature

White holes

A WHITE hole is considered to be any object which emerges through a past 'Schwarzschild sphere'. Recently Gribbin¹ has given a popular account of white holes. These objects were first considered by Novikov² and by Ne'eman³ as 'lagging cores', parts of the initial big bang long delayed in their expansion. Szekeres⁴ discovered that a canonical white hole was unstable due to a blueshift divergence along the past 'Schwarzschild sphere'. Eardley⁵ argued that catastrophic accretion would reconverge outgoing photons so that a white hole would inevitably appear to an external observer as a black hole. Here we show that observable white holes ought to be considered as undelayed local inhomogeneities in the big bang, and we distinguish those white holes which we are most likely to 'see' today.

Although the formal stability of a white hole simply requires a finite delay time⁶, its actual survival or death can be decided by the magnitude of the frequency shift on the 'Schwarzschild sphere'. To calculate this in a reasonably general situation let us consider a totally geodesic 2-surface Γ so that

$$ds^2_\Gamma = -f(r)dt^2 + f^{-1}(r)dr^2, \quad (1)$$

and suppose that Γ contains a bifurcate Killing horizon⁷ H at $r=a$ (that is, an horizon which crosses at some point p on Γ). As a must be a simple root of f , $f(r) = (r-a)h(r)$ where $h(a) \neq 0$ and finite, and the surface gravity⁸ of H is given by

$$\kappa = f'(a)/2 = h(a)/2$$

To exhibit a regular covering of Γ about H we make the unique partial fraction decomposition

$$1/f(r) = 1/2\kappa(r-a) + g(r)/h(r), \quad (2)$$

note that $g(a)/h(a) \neq 0$ and finite, and introduce 'double null' coordinates by the relationship

$$uv = \pm \frac{(r-a)}{a} \exp \left[2\kappa \int \frac{g(r)}{h(r)} dr \right] \quad (3)$$

and

$$\left| \frac{v}{u} \right| = \exp(2\kappa t). \quad (4)$$

The line element (1) then takes the form

$$ds^2_\Gamma = K(r)du dv, \quad (5)$$

with

$$K(r) = \pm \frac{ah(r)}{\kappa^2} \exp \left[-2\kappa \int \frac{g(r)}{h(r)} dr \right] \quad (6)$$

With the integration constant set to zero in equations (3) and (6) the factor a determines the scale of u and v . The choice of sign

determines the orientation of the u - v axis. Summarising the above we can say that every totally geodesic 2-surface Γ which contains a bifurcate Killing horizon admits a u , v chart which gives a regular covering of a subregion of Γ containing the horizon, such that

$$ds^2_\Gamma = -\frac{\xi^2}{\kappa^2} \frac{du dv}{uv}, \quad (7)$$

where κ is the surface gravity and ξ the Killing vector.

The Lagrangian associated with the metric (5) is given by

$$2\mathcal{L} = K(r) \dot{u} \dot{v} = \begin{cases} -1 & \text{Timelike geodesics (TL); } \dot{\tau} \equiv d\tau/d\lambda \\ 0 & \text{Null geodesics (N); } \dot{\lambda} \equiv d\lambda/d\lambda \end{cases}, \quad (8)$$

where τ and λ are, respectively, the proper time and an affine parameter, and for TL geodesics on Γ we have⁹

$$\dot{r}^2 = \gamma^2 - f(r), \quad \gamma = \text{const.} \quad (9)$$

In the r , t coordinates $\gamma \equiv -\partial\mathcal{L}/\partial\dot{t} = f(r)\dot{t}$. Here we choose the negative of equations (3) and (6), take $\kappa > 0$, distinguish the branches of H by H_1 for $u = 0$ and H_2 for $v = 0$, and choose a future orientation on Γ . Then the neighbourhood of H can be represented as in Fig. 1 where we have distinguished future directed TL geodesics by the sign of γ (TL geodesics with $\gamma = 0$ are the only TL geodesics which pass through the bifurcation point p).

We now calculate the frequency shifts detected on H by TL geodesics of Γ . With the emitter designated by $\hat{e}$ and the observer by $\hat{o}$, the generalised frequency shift formula states that $1+z = \text{emitted frequency/observed frequency} = (\mathbf{w} \cdot \mathbf{k})_{\hat{e}}/(\mathbf{w} \cdot \mathbf{k})_{\hat{o}}$, where $\mathbf{w}$ is the TL geodesic tangent and $\mathbf{k}$ the null geodesic tangent so that with the metric (5) and equation (8) we calculate

$$1+z = \dot{u}_o/\dot{u}_e \text{ for } \dot{v}_N = 0$$

and

$$1+z = \dot{v}_o/\dot{v}_e \text{ for } \dot{u}_N = 0. \quad (10)$$

For $\gamma \neq 0$ we differentiate equation (3) and use equations (9) and (10) to find

$$(1+z)_{H_1} = \gamma_e v_o/\gamma_o u_e$$

and

$$(1+z)_{H_2} = \gamma_e u_o/\gamma_o v_e. \quad (11)$$

From equation (4) it follows that $v = u \exp(2\kappa t^*)$ along a $\gamma = 0$ TL geodesic where t^* is a finite constant. With the aid of equations

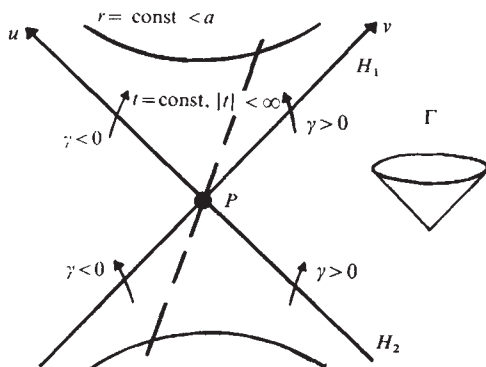


Fig. 1 'Kruskal' diagram for the 2-surface Γ in the neighbourhood of the Killing horizon H . Null geodesics on Γ are represented by constant u or v lines.

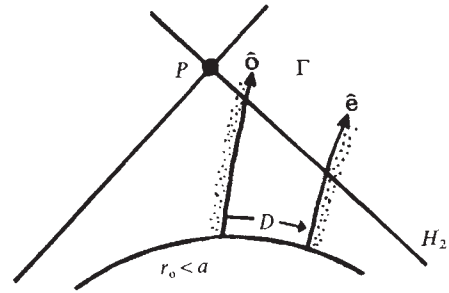


Fig. 2 A white hole $\hat{o}$ as a delayed part of the cosmic background field $\hat{e}$. The delay D is the ' t -measure' along r_0 from $\hat{o}$ to $\hat{e}$. To the left of $\hat{o}$ and to the right of $\hat{e}$ the geometry is that of a suitable matter distribution.

(6) and (9) we find that for $\gamma_e = 0 \neq \gamma_o$

$$1+z_{H_1} = \frac{v_o}{\gamma_o} \left(\frac{\kappa a}{2} \right)^{1/2} \exp[-\kappa(R+t^*)]$$

and

$$1+z_{H_2} = -\frac{u_o}{\gamma_o} \left(\frac{\kappa a}{2} \right)^{1/2} \exp[-\kappa(R-t^*)] \quad (12)$$

where

$$R \equiv \left[\frac{g(r)}{h(r)} dr \right]_{r=a} \quad (13)$$

According to equations (12), a blueshift divergence develops to the future of p on H as $\kappa \rightarrow 0$. For $\gamma_o = 0 \neq \gamma_e$ we obtain the reciprocals of equations (12) with $\hat{o}$ replaced by $\hat{e}$. The transformation

$$u' \equiv u \exp(\kappa t_o)$$

and

$$t' \equiv t \exp(-\kappa t_o), \quad (14)$$

where t_o is a finite constant, gives $t' = t - t_o$ according to equation (4). Since a global transformation (that is equation (14), applied to both $\hat{e}$ and $\hat{o}$) leaves $(1+z)_H$ unchanged, we set $t^* = 0$ in equations (12). A shift $t \rightarrow t - t_1$ along $\hat{e}$ (or equivalently $t \rightarrow t - t_2$ along $\hat{o}$ with $t_2 = -t_1$) gives

$$(1+z)_{H_1} \rightarrow (1+z)_{H_1} \exp(\kappa t_1)$$

and

$$(1+z)_{H_2} \rightarrow (1+z)_{H_2} \exp(-\kappa t_1) \quad (15)$$

so that $h v_o \propto \exp(-\kappa t_1)$ along H_1 whereas $h v_o \propto \exp(\kappa t_1)$ along H_2 . This should not be confused with the fact that $h v_o \propto \exp(-\kappa t)$ for Killing emission just above H_2 with $u < 0$.

Let $\hat{o}$ represent the boundary of a white hole and $\hat{e}$ the boundary of the external universe ('cosmic background field'). Here, for simplicity, let $\gamma \geq 0$, consider $\gamma_e > \gamma_o$ and concentrate on that portion of H_2 with $u \leq 0$. Suppose there is a curvature singularity at $r_0 < a$ on Γ , and let D be the ' t -measure' along r_0 from $\hat{o}$ to $\hat{e}$, that is the 'delay', as shown in Fig. 2.

Representing the value of $(1+z)_{H_2}$ for $D = 0$ by $(1+z)_{H_2}^0$ so that from equation (15) $(1+z)_{H_2} = (1+z)_{H_2}^0 \exp(-\kappa D)$ or $h v_o \propto \exp(\kappa D)$ on H_2 , κD must be small otherwise $\hat{o}$ would receive a tremendous blast of energy (more precisely, the geometry of Γ would distort). This means that a white hole cannot bud forth from r_0 today. But does this mean that we cannot observe a white hole today? Indeed we can. First, suppose that κD is small (if r_0 is not 'naked' in the sense that 'uncontrollable' information can propagate into Γ , we must set $D = 0$). It is evident from Fig. 1 that the proper time τ_e during which $\hat{e}$ (which here we assume to be open in the sense that it does not cross H_1 with $v > 0$) observes the white hole $\hat{o}$ to be in expansion near H increases as γ_o becomes

small. Calculations show that this relation is very sharp so $\gamma_0 \approx 0$ for reasonably large values of τ_e (τ_e is arbitrarily large for all finite κ with $\gamma_0 \leq 0$). Note that with $D \approx 0$ there is no energy blast from $\dot{e}$. For example, in the Schwarzschild spacetime with $D = \gamma_0 = 0$ and $\gamma_e \geq 1$ ('open') it follows that $(1+z)_p > 2\sqrt{e}$.

We conclude that white holes must be considered as undelayed local inhomogeneities in the big bang. The ones we are most likely to 'see' today do not expand beyond their 'Schwarzschild spheres' into our space and have, in fact, always been visible. Other white holes have either recollapsed (and hence appear as black holes) or have evolved far from their 'Schwarzschild spheres' and hence would not generally be considered as 'white holes'.

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Photometric confirmation of the Encke division in Saturn's ring A

APPROXIMATELY once every 15 yr, it is possible to observe Saturn's satellite Iapetus passing through the shadow of the rings. Observations of such events are of great value to studies of the optical properties of the rings, as ground-based observations of reflected light are limited to a small range of phase angles near zero ($|\phi| < 6^\circ$), and eclipses give transmission data at $\phi = 180^\circ$. During the current apparition, Iapetus experiences two eclipses, the first having occurred on 19-20 October 1977, and the second 7-8 January 1978. The observed brightness of Iapetus during eclipse represents a spatial convolution of light from the limb-darkened disk of the Sun with the transmission of the rings and the non-uniform reflectivity over the disk of Iapetus. We have examined this physical model through a computer simulation of the eclipse and use this to give here photometric confirmation of the Encke division in ring A.

The October event was observed with a two-channel sky-chopping photometer at the 154 cm Mt Lemmon reflector of the University of Arizona. Simultaneous pulse-counter output from the red ($\lambda \sim 6,000 - \sim 9,000 \text{ \AA}$) and blue ($\lambda \sim 4,000 - \sim 5,000 \text{ \AA}$) channels was integrated for 10-ms intervals alternatively on sky and on Iapetus. Data recording was begun at 11:30 UT and extended to 12:56 UT, well into morning twilight. Observations of Titan were made periodically to calibrate atmospheric extinction. The data, expressed in stellar magnitudes, with zero being the un-eclipsed brightness, are shown averaged over 1-min intervals in Fig. 1. The presence of a division in the outer portion of ring A is demonstrated by the temporary brightening in the lightcurve.

In the computer eclipse simulation we used a solar limb-darkening coefficient of $u_1 = 0.65$ in the blue wavelength region, and Iapetus was treated as two limb-darkened hemispheres of radii 800 km (ref. 2), with the trailing hemisphere brighter by a factor of 7 than the preceding hemisphere³. A series of ringlet models⁴ was then used as trial ring optical thickness profiles. The ring profile proved to be the dominant input parameter, as the model was largely

insensitive to plausible variations in the solar brightness distribution, Iapetus' reflectance, or eclipse geometry. The best fit ring model is given in Table 1 and is drawn in Fig. 1, with the eclipse lightcurve generated from this ring model. Several conclusions may be drawn from the good agreement with the data. (1) A division in the outer part of ring A is certainly present. The division is centred at a distance of $3,900 \pm 100$ km from the outer edge of ring A. The measurements of Dollfus⁵ and Reese⁶ give a radius of $136,600 \pm 200$ km for the outer edge of ring A which places the division at a radius of 132,700 km, with an uncertainty of at least several hundred km. (2) The average normal optical thickness of the outer portion of ring A is 0.36 ± 0.01 . This spatial average represents the total transmission of the rings over a characteristic diameter of 10^3 km. (3) The observed division has transmission equal to a clear gap of 325 ± 25 km in the $\tau = 0.36$ ringlet. We have assumed a partially filled gap with a width of 600 km for our model, while a width of up to 750 km is possible if the optical depth is increased further. (4) The outer boundary of ring A is abrupt. Experience with the simulation program suggests that the data would reveal any outer boundary transition region wider than about 200 km. Higher resolution is precluded by the 3,000 km effective diameter of the solar image at the distance of the rings as seen from Iapetus. (5) The ephemeris provided by A. W. Harris (personal communication) is quite accurate. The time at which the outer boundary of the ring lay between the centres of Iapetus and the Sun was $12:24 \pm 0.3$ UT, while the predicted immersion was 12:25 UT.

Telescopic investigations of the ring system in optimum conditions have previously revealed the presence of a faint division in ring A which is commonly called the Encke division. Although traditionally located about one-third of the distance from the outer boundary of ring A to its inner

Fig. 1 Iapetus eclipse data with best-fit ring model and resulting eclipse lightcurve. Plotted points are 1-min averages of the blue data expressed in stellar magnitudes. Ring optical thickness τ at the position of the central ray of the eclipse is plotted for the three-ringlet model, with ring radius indicated on the upper scale. The smooth curved line results from the convolution of the ring model with a limb-darkened solar disk and a two-hemisphere albedo model for Iapetus. The red data (not shown here) indicate that the event was essentially independent of colour.

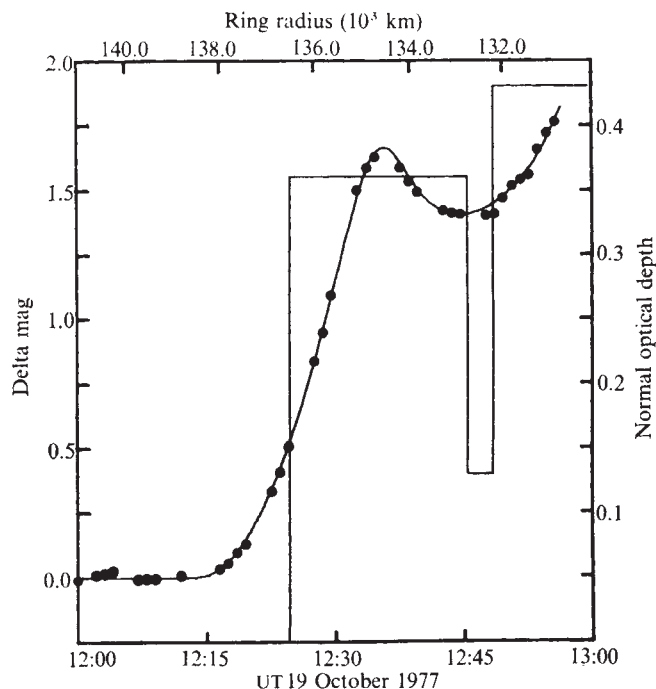


Table 1 Ring model

Ringlet boundary (km)	Normal optical depth τ^*
136,600	0.36
133,000	0.13
132,400	0.43
< 131,000	

* $\tau = -|\sin B'| \ln T$, where T is the observed ring transmission and B' is the planetocentric latitude of the Sun.

edge⁷ more recent quantitative measurements^{5,6} place the major division in ring A about one-fifth of its width from the outer edge. This agrees with the location of the division contained in the eclipse data, which may, therefore, be identified with the Encke division.

The location of the division is near to the radius at which a particle orbits Saturn with a period $5/3$ that of the satellite Mimas. The radius of period commensurability is 123,100 km (ref. 8), while the observed radius for the Encke division is a few hundred km greater. If satellite resonances act as barriers to outward migration of material in ring A, the corresponding divisions would be slightly displaced outward from the resonance position. Another period commensurability in this region would be the $4/3$ resonance with a satellite having a period of 0.76 d. This value is close to the orbital period of Janus, while additional inner satellites may also exist with similar periods⁹. Thus, the location of the Encke division is consistent with the interpretation of ring divisions in terms of three-body interactions.

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Expected shape distribution of asteroids obtained from laboratory impact experiments

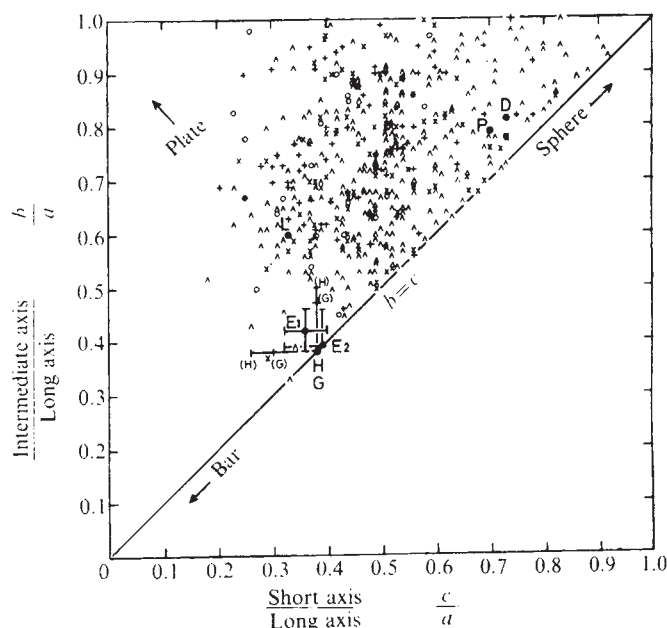
MANY asteroids are known to have irregular shapes from their periodical light curves. These irregularities in shape are thought to be due to destruction in asteroidal collisions which occur at a mean relative velocity of 5 km s^{-1} . None of these asteroids has been directly pictured and the actual shape distribution of them is unknown. We examine here the shape of the fragments produced in laboratory high-velocity impact experiments between two solid bodies, and we find that the obtained shape distribution may be applied to asteroids of radius less than a few km.

In our experiments, cylindrical polycarbonate projectiles of mass 0.37 g and diameter 0.8 cm were impacted into cubic basalt block targets about 5 cm in size and with density 2.7 g cm^{-3} . The impact velocity range was

$1\text{--}4 \text{ km s}^{-1}$, obtained by a light gas gun¹. The fragments produced in each impact were graded for size with sieves. The shapes of the fragments $> 4 \text{ mm}$ from the selected two targets were measured. Projectile velocity and target size of these two impacts were 2.6 km s^{-1} , about 5 cm (I1) and 3.7 km s^{-1} , about 5.2 cm (I2). Both targets were catastrophically destroyed and left central cores and smaller fragments (core-type impact¹). The number of fragments $> 4 \text{ mm}$ in each impact was 275 for (I1) and 444 for (I2). The shape parameters of a , b and c were defined in the following way. The short axis length c was determined as the minimum among the distances between a pair of parallel planes making contact with a fragment. In a similar way, the intermediate axis length, b , was defined as the minimum among the distances between each pair of parallel planes which make contact with the fragment and are normal to the planes chosen to define the short axis length c . The long axis length a is the distance between two sheets of the planes normal to both pairs of planes chosen to determine c and b ($a \geq b \geq c$). The measurements were made manually with the use of slide calipers, and the accuracy was considered to be $< 0.05\text{--}0.01 \text{ cm}$.

It is remarkable in the c/a against b/a diagram (Fig. 1) that all points are distributed in the region of $c/a \geq 0.2$, and markedly bar-like fragments are not found. Also noted is that there are very few sphere-like fragments. From the histogram of b/a and c/a for the 719 fragments $> 4 \text{ mm}$ (I1 and I2), average values of b/a and c/a are 0.50 and 0.73, respectively. This means that the average ratio of $a:b:c$ is given to a good approximation by a very simple ratio of $2:\sqrt{2}:1$. Figure 1 shows that there seems to be no systematic change in the shape distribution for different size ranges of fragments. There is no well-established theoretical basis to which this distribution is applicable for much larger objects, but it may safely be used as long as the internal stress in a body is less than the yield stress of the constituent material of the body.

Fig. 1 Shape distribution of the fragments produced in a single impact (I1) (size of the basalt block target, 5.2 cm; polycarbonate projectile mass, 0.37 g; impact velocity, 3.7 km s^{-1}) and the shape parameters of martian satellites and asteroids. The shape parameters of these minor celestial bodies were adopted from ref. 5 for 39 Laetitia (L), refs 6 (E_1) and 2 (E_2) for 433 Eros, ref. 3 for 624 Hektor (H), ref. 4 for 1620 Geographos (G) and ref. 9 for Phobos (P) and Deimos (D). Fragment size (mm): $\times$, 4–6; $\times$, 6–6.5; $+$, 6.5–9; $\circ$, 9–15.



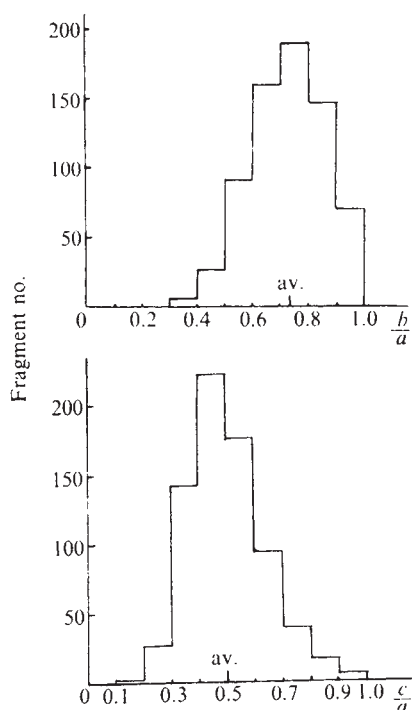


Fig. 2 Histogram for b/a and c/a . av., average value. The average ratio of $a:b:c$ is approximately given by $2:\sqrt{2}:1$.

Apart from the statistical errors, there were no appreciable differences between the results obtained at impact velocities of 2.6 km s^{-1} (11) and 3.7 km s^{-1} (12). This means that the shape distribution will also apply to the fragments produced by an impact of about 5 km s^{-1} , which is the mean relative collision velocity of the asteroids. The obtained shape distribution may be considered to be widely applicable to the fragments from the impacted brittle materials, and to be almost independent of impact velocity, size of the fragments (but under the above-mentioned limitation) and perhaps also the class of impact¹.

It is interesting to compare the experimentally determined shape distribution with the shapes of the minor celestial bodies. Apart from the directly pictured bodies, Phobos and Deimos, asteroid shapes are determined from their light curves. The asteroid models are usually a cylindrical bar with semi-spherical caps at both ends⁷⁻⁹, or a triaxial ellipsoid^{10,11}, and a few shape parameters appearing in each model are modified to give the best fit with the observations. Therefore, it must be assumed that the individual asteroid shape is not always exactly determined. Figure 1 indicates that the points for Phobos and Deimos and the four asteroids whose shape parameters are available are all in the region of $c/a \geq 0.2$, even if the uncertainties due to adopted models are included. Phobos and Deimos have nearly average shapes as collisional fragments. On the other hand, the cited four asteroids have c/a values near the limiting one. These asteroids are known to have considerable amplitude of brightness variations and are expected to be those of the highest class of irregularity; this is confirmed from the comparison with our experimental data.

Our shape distribution is considered to represent that for rock-like (not metallic) asteroids of sufficiently small size for the internal stress to be less than the yielding strength of the constituent material. Highly irregular rocky objects of radius $> 300\text{--}500 \text{ km}$ will gradually change their shapes into spheres⁷; the large asteroids may not have been collisionally fragmented. (It is known that the largest asteroids have near-spherical shapes; for example, $b/a, c/a \approx 0.8\text{--}0.9$ for Vesta⁸.) For these reasons,

the obtained shape distribution is applicable to the asteroids of radius less than a few km.

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Non-stoichiometry and dielectric properties

A LONG-STANDING problem concerning the structural properties of non-stoichiometric phases is the factor which determines whether point or extended defects are formed. We discuss this problem using results of exact lattice energy calculations. We find that cation relaxation plays a crucial role in stabilising extended defects; and we show that this explains an empirical correlation, established by Tilley¹, between the dielectric properties and defect structure of non-stoichiometric compounds.

Tilley considered the formation of extended defects; these occur in a restricted range of non-stoichiometric compounds (for example, in TiO_{2-x} where shear planes are observed), while point defect structures are formed in the majority of disordered crystals (such as Fe_{1-x}O (ref. 2), UO_{2+x} (ref. 3) and SnO_{2-x} (D. Armytage and B. E. F. Fender, personal communication)). Tilley showed that extended defect formation is strongly correlated with high values of the static dielectric constant, ϵ_0 , of the crystal. Shear planes are, for example, observed in TiO_2 and WO_3 , where ϵ_0 has the values of ~ 170 and $1,000$, respectively. In the materials where point defects are formed, the dielectric constants are much lower, thus in FeO and SnO_2 , ϵ_0 is ~ 13 and 14 , respectively. The contrast between TiO_2 and SnO_2 is particularly interesting, as the materials are isostructural with similar lattice parameters, and the difference in dielectric properties is probably the most marked macroscopic distinction between the two compounds.

Tilley¹, however, did not formulate a theoretical account of his empirical correlation, although he speculated on the importance on shear plane stabilisation of soft phonon modes in highly polar materials. Here we show that there is a simple but precise atomistic explanation of the observed correlation. We use accurate calculations of shear plane energetics, and our results explain the puzzling contrast, mentioned above, between the behaviour of SnO_{2-x} and TiO_{2-x} .

Shear plane formation involves a modification of the topology of the crystal, which, although eliminating point defects, results in shorter cation-cation separations. Thus, in TiO_{2-x} edge sharing between oxygen octahedra is replaced in the shear plane by face sharing; this reduces the oxygen-to-metal ratio in the crystal but brings the titanium cations in the face-shared octahedra closer together. Clearly, the balance between extended and point defect formation will be largely determined by the relative magnitudes of the point defect formation energy and

Table 1 Relaxation energies accompanying shear plane formation

	Ti ₅ O ₉	Sn ₅ O ₉
Relaxation energy (eV) due to cation displacement (in polarisable lattice)	9.3	0.4
Ionic polarisation energy (eV) of observed structure*	4.3	1.3

*Difference between shear plane energy for polarisable and rigid ion lattice with Ti₅O₉ structure.

the increased cation-cation repulsion resulting from the formation of shear plane. We suggest that in general, the inter-cation repulsive term outweighs the point defect energy and thus extended defects are not stable. However, for crystals with large values of the static dielectric constant, this repulsive energy is reduced and shear plane formation becomes favoured.

We shall now explain the latter phenomenon using a detailed atomistic model for the shear plane. This approach contrasts with previous discussions⁴ that used a continuum model which gives the following simple expression for the increased cation repulsion energy, ΔE , accompanying shear plane formation:

$$\Delta E = \frac{q^2}{\epsilon_0} \left(\frac{1}{r_s} - \frac{1}{r_0} \right) \quad (1)$$

where q is the cation charge, r_s the nearest neighbour cation separation across the plane, and r_0 the nearest neighbour cation separation in the ideal solid. The equation predicts a reduction with increased static dielectric constant of the unfavourable cation repulsion energy across the plane. The expression is, however, inaccurate: even with a continuum model it is certainly unsatisfactory to use a value of ϵ_0 appropriate for bulk stoichiometric TiO₂, for interactions within the shear plane, owing to the extensive structural modification occurring in this region (the local order within the shear plane in TiO₂ is indeed close to that of the corundum structure). Moreover, using equation (1) as an explanation of shear plane stabilisation in highly polar materials is misleading; it implies that cation repulsions are screened by polarisation of the intervening atoms in the plane. We shall show that this is not the case by demonstrating that relaxations of the cations themselves provide the major stabilising effect.

Continuum models are clearly inadequate for evaluating electrostatic interactions within shear planes. We now analyse shear plane stabilisation in TiO₂ based on atomistic calculations of the lattice energy terms accompanying the formation of the shear plane. We use the PLUTO program⁵ which sums both short range and Coulomb contributions to the lattice energy exactly. Any inaccuracies in the calculation will arise from inadequacies in the lattice potential. However, the potentials used here are derived by well-established semi-empirical methods, and they successfully predict dielectric, elastic and structural properties of the crystal (further details are available elsewhere⁶).

Our potentials are based on a fully ionic model—clearly an approximation for TiO₂. At present, however, there seems to be no simple way of including covalence in our calculations. But, the adequacy of the ionic model for our present purposes is supported by the success of our calculations in achieving reasonable quantitative agreement with experimentally determined shear plane energies, as discussed below.

Calculations on isolated shear planes are, at present, not possible. Our study was performed, therefore, on the Ti₅O₉ structure which contains regularly spaced planes. This compound has the advantage that accurate structural data on the cation positions in the vicinity of the plane are available. The calculations are all performed with the reduced Ti³⁺ ions in the nearest neighbour position with respect to the shear plane. Of course, our calculated shear plane energy must include a contribution from defect interactions. However, as the shear plane energy we deduce for TiO₂ is within 4% of that obtained by a

Born-Haber analysis of the thermochemical data of Picard and Gerdanian⁸, this factor does not seriously affect our present argument.

In the Ti₅O₉ structure, large cation relaxations ($\sim 0.3\text{\AA}$) are observed in the region of the shear plane. To estimate the stabilising effect of these titanium displacements, we calculated the energy of the observed structure, and that of an 'ideal' structure in which the cation relaxations are removed by returning the Ti ions to the centre of their octahedra. The resulting calculated relaxation energy (per pair of Ti ions) is given in Table 1. Also given is the relaxation energy accompanying the atomic relaxations around a hypothetical shear plane in reduced SnO_{2-x}. These were obtained in calculations on the hypothetical Sn₅O₉ structure, which was assumed to be identical to Ti₅O₉, except for its lattice parameter. Our SnO₂ potential was derived using the same techniques as applied to TiO₂.

We also calculated the energy for the structure including cation relaxations, but in which ionic polarisation was not allowed. Comparison with our other calculations allowed us to estimate the effect of ionic as well as displacement polarisation on shear plane stability.

Our results, summarised in Table 1, demonstrate three important points. First, the stabilising effect of cation relaxation is large for shear planes in TiO₂. Moreover, as there is evidence that there is a fine energy balance between shear planes and point defects in TiO₂ (shear planes, it seems, dissociate into point defects at very low deviations from stoichiometry⁹), it is clear that in this material shear plane formation would be energetically prohibitive without these large relaxations. Our calculated relaxation energy assumes no electronic screening of electrostatic interactions within the plane. As TiO_{2-x} is known to be an electrical conductor, this may lead to some exaggeration of the relaxation term. Such effects are, however, unlikely to affect our qualitative arguments.

Second, we find that in SnO₂, the relaxation energy is more than an order of magnitude smaller; cation displacements cannot stabilise shear planes to the same extent as in TiO₂. Also, we investigated the possible valence effect arising from reduction of tetravalent to divalent tin (in contrast to titanium which is reduced to the trivalent state). This effect, we found, could lead only to enhancement of the shear plane energy in SnO₂. The failure to observe shear planes in TiO₂ must therefore be attributed to the relaxation effect, that is, large cation relaxations play an essential role in stabilising shear planes. In TiO₂ such relaxations are far more strongly favoured energetically than in SnO₂.

Third is the significant role of ionic polarisation; for TiO₂ this effect stabilises the shear plane by $\sim 4\text{ eV}$, which is less, however, than the effect discussed above due to displacement polarisation.

Our identification of the essential role of cation relaxation in stabilising shear planes provides a ready explanation of Tilley's empirical correlation of shear plane formation with dielectric properties. Large cation displacements may occur in materials with a high displacement polarisability, which also leads to a high dielectric constant. We emphasise that it is not the high dielectric constant which results in shear plane stabilisation, as might be implied by equation (1). The dielectric constant is correlated with shear plane formation because both are determined by the same factor, that is, the magnitude of the displacement polarisability, which may in turn be related to the metal-oxygen force constants. The latter also largely determine the magnitude of elastic constants and phonon frequencies. Correlations could therefore, be sought with equal validity, between shear plane formation and the soft phonon mode observed in TiO₂ (ref. 10), which can be assigned to displacement of the Ti⁴⁺ ions against the oxygen octahedra, and which must therefore be attributed to the nature of the metal-oxygen potential. Again, however, it would be incorrect to argue that the soft phonon mode 'caused' the shear plane to be stable; rather the soft phonon mode arises from the same fundamental factor responsible for the stabilisation of the shear plane.

We have thus identified cation relaxation as an essential factor in shear plane stabilisation and shown how this correlates with bulk properties of the lattice, particularly the dielectric constant. We have argued that dielectric properties play no direct role in stabilising shear planes, in particular, arguments based on a continuum formalism suggest that the dominant effect of screening of the Coulomb interaction across the shear plane, are misleading. Our atomistic calculations therefore provide a valuable insight into the problem of shear plane formation. Their power and scope will enable us to extend our investigation to more complex structures in a comprehensive survey of shear plane stability.

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Climatic signals in British Isles tree-ring chronologies

INTERPRETATION of proxy records of past environmental conditions derived from dated geological or biological materials is of great importance for the extension of the climatic record^{1,2}. Precisely dated, replicated tree-ring series have been particularly useful as they provide records (usually ring-widths) dated to the individual year, for hundreds or thousands of years. Each such series, or chronology, is derived from a particular known location. Thus a network of such chronologies may be developed for a particular region and used as a proxy record of spatial and temporal climatic variations. This has been achieved in North America by Fritts *et al.*³⁻⁵, using principally chronologies from semi-arid areas or from near altitudinal or polar tree-lines. We report evidence here that tree-ring chronologies from sites in the British Isles will provide suitable proxy records for the reconstruction of historical temporal and spatial variation of climate.

For a tree-ring chronology to be of value in climatic reconstruction it should have a clear and discernible relationship to climate. The tree-site complex may be considered as a transducer, with external environmental conditions as inputs and a series of changing annual ring-widths as output. For the present purpose the best situation is that where, considering a record of past climate as signal, the signal-to-noise ratio in the series derived from annual ring-widths is greatest. To achieve this it is necessary, *inter alia*, to use chronologies based on adequate replication from which non-climatic movement has been removed as far as possible⁶. This noise may derive from ontogeny, management, stand development, other biota or other factors affecting ring-width.

Our results are based on an analysis of the relationship between three such chronologies (Table 1) and meteorological data. All three chronologies are for oaks, the most commonly used dendrochronological material in Europe^{6,7}. Every ring on each radius measured has been crossdated absolutely by methods described elsewhere^{8,9}. Each chronology is examined in relation to mean monthly temperature (monthly mean maximum for Raehills) and monthly precipitation for a nearby meteorological station (Table 1). The response function approach³ has been

used to examine the strength and nature of the climatic signal in these three chronologies. The climatic data are reduced to eigenvectors and used to estimate the elements of the tree-ring chronology thus:

$${}_1\hat{\mathbf{P}}_n = {}_1\mathbf{R}_p\mathbf{E}'_m\mathbf{F}_n \quad (1)$$

where ${}_1\hat{\mathbf{P}}_n$ is a row vector of estimates of ring-width indices, ${}_m\mathbf{E}'_p$ the transpose of the eigenvector matrix of climatic data, ${}_m\mathbf{F}_n$ the original climatic data for m variables and n years and ${}_1\mathbf{R}_p$ a row vector of p significant multiple regression coefficients between ring-width indices and the eigenvectors of climatic data. A row vector ${}_1\mathbf{T}_m$ may be derived which has elements representing the magnitude of growth response to each climatic variable, thus

$${}_1\mathbf{T}_m = {}_1\mathbf{R}_p\mathbf{E}'_m \quad (2)$$

This vector, the response function, does not take account of the autocorrelation found in such tree-ring chronologies^{9,10}. To do this, ring-width indices for the three previous years are included in the regression analysis as if they were orthogonal³.

The response functions for the three British Isles oak chronologies (Fig. 1) are based on a 14-month year for mean monthly temperature and monthly precipitation which commences in June of the year before growth and ends in July of the year of growth. Physiological evidence suggests that this largely contains the periods of latewood formation in both years¹¹. An extension to a 16-month year ending in September of the year of growth increased variance accounted for by the Rostrevor response function by only 1.5%. It is evident that the three response functions differ from one another (Fig. 1). For example, whilst all three sites show significantly positive temperature elements in the October before growth, only Maentwrog and Rostrevor show such a response in the July before growth and the April of the year of growth. This is normally the month of first bud opening and of the commencement of earlywood vessel formation¹¹. Maentwrog and Rostrevor show significantly negative temperature elements in the December before growth, whilst Raehills does not. Rostrevor shows five significantly positive precipitation elements, whilst Raehills has one positive and two negative, and Maentwrog two significantly negative precipitation elements. These differences in the response functions for the three chronologies signify that the transducer comprising the tree-site complex at each site has a characteristic response to climate. This arises from the particular combination of genetic, physiological and ecological factors determining the interaction between tree growth and site conditions¹². These differences between the response functions may be valuable for climatic reconstruction. By using chronologies recording different elements of climate a greater range of climatic variables will be reconstructable.

Table 1 Details of tree-ring chronologies and meteorological data

Chronology Location	Rostrevor ¹⁰ County Down, Northern Ireland	Raehills* Dumfriesshire Scotland	Maentwrog ⁹ Gwynedd, Wales
Altitude	< 160m OD	170–200m OD	< 100m OD
Slope	Steep	Steep	Steep
Aspect	South-west	South-east	South
Substrate	Acidic, stony soil	Clayey, stony soil	Acidic soil
Species	<i>Quercus petraea</i>	<i>Quercus</i> sp undetermined	<i>Quercus petraea</i>
Number of trees	18	10	35
Number of radii	36	10	70
Station for rainfall	Silent Valley	Dumfries	Aberystwyth
Station for temperature	Armagh	Dumfries	Aberystwyth
Period of analysis	1924–68	1910–55	1907–67

All three chronologies were compiled from measurements standardised using a polynomial curve-fit indexing procedure.

*M. Baillie, unpublished data.

The calculation of chronology variance accountable by the climatic elements of the response functions (Table 2) shows there to be a clear climatic signal in the tree-ring data. All three chronologies show a greater percentage of variance related to climate than the 26% reported for an oak stand in Schleswig-Holstein¹³. The Rostrevor and Raehills chronologies show climatic signal-to-noise ratios similar in magnitude to those found in the semi-arid American South West¹². The differences between the Maentwrog chronology and the other two may be due, in part, to the distance of the site from the meteorological station. If a Maentwrog chronology compiled from measurements passed through a high-pass digital filter blocking variance with wavelengths greater than eight years¹⁴ is examined, differences from the unfiltered Maentwrog chronology emerge. In particular the proportion of chronology variance related to previous growth is reduced from 31% to 19% whilst that related to climate is increased from 34% to 40%. This indicates that a stronger climatic signal is present in the high frequency component than in the unfiltered chronology.

The application of the response function approach to these British Isles oak chronologies has demonstrated their potential as proxy records of temporal and spatial climatic change. The unmodified technique seems to be of such general application that it can be used in widely differing conditions. Of the weaknesses of the approach, outlined by Fritts⁵, the one relating to frequency variations in chronology-climatic relations seems to be of special significance here. It is possible that a close examination of these variations will be of particular value in optimising the use of this powerful technique with the kind of material discussed here. Nevertheless, the detection of clear

Table 2 % chronology variance accounted for by response functions

	Climate	Previous growth	Total
Rostrevor	64	14	78
Raehills	61	17	78
Maentwrog	34	31	65

characteristic climatic signals in these three chronologies, coupled with the established capability for building 250-year or longer chronologies^{9,10} indicates that the use of such chronologies as proxy records of temporal and spatial climatic change in the region containing the British Isles is feasible. Thus it should be possible to produce maps of annual or even seasonal climatic anomalies for that region as has been done for the North Pacific-Western North America sector¹. Such maps would provide a significantly more detailed picture of climate in the last 250 years than is now available.

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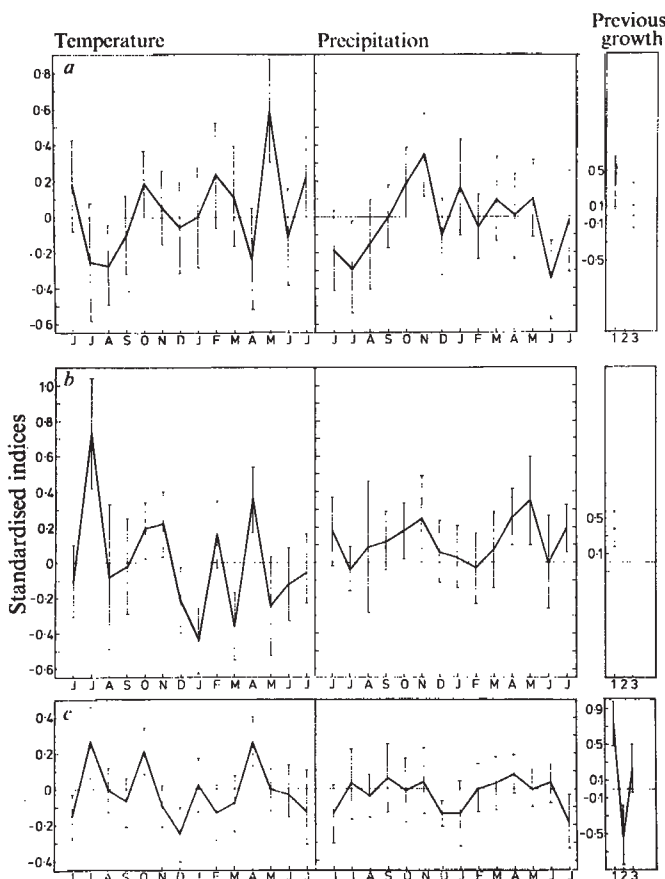


Fig. 1 Response functions for the three oak chronologies referred to in Table 1. The elements for temperature and precipitation for each of 14 months are shown with their 95% confidence limits. Also shown are the elements for three years' previous growth, along with their confidence limits. A positive element shows a direct relationship between the variable in question and ring-width index. A negative element shows an inverse relationship. *a*, Raehills; *b*, Rostrevor; *c*, Maentwrog.

Deep seismic zone as an upper mantle reflector of body waves

THE deep seismic zone associated with the Izu-Bonin arc dips at about 40° towards the south-west beneath the Dodaira Observatory, Japan (Fig. 1). At this observatory an unexplained phase with apparent velocity of 16.5 km s⁻¹ was observed 10-20 s after the first arrival for earthquakes in the Ryukyu Islands. No corresponding phase has been

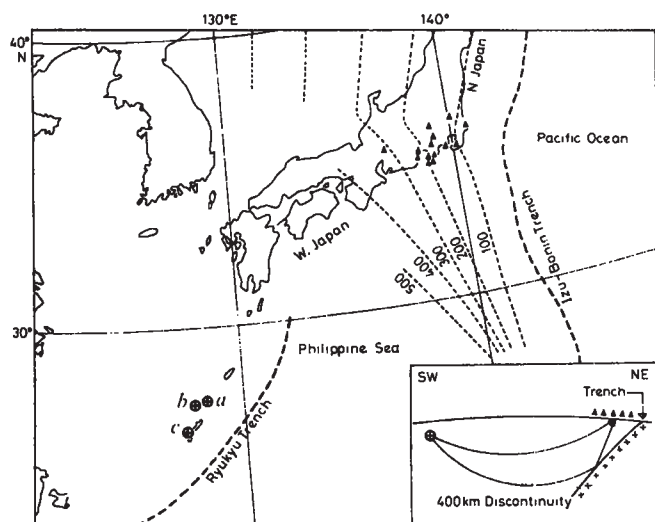


Fig. 1 Izu-Bonin deep seismic zone contoured in 100 km depth, the epicentres of the related earthquakes in the Ryukyu Islands and the seismic stations (▲) used in the analysis. *a*, 26 October 1972; *b*, 17 March 1974; *c*, 7 January 1976. Inset: schematic ray diagrams explaining the mechanism of the X phase.

found for earthquakes of different azimuths. This unexpected phase can be explained by post-critical reflection of P waves at the deep seismic zone after strong focusing by the 400-km transition zone: this zone of reflection is apparently sharp, of the order of 10 km in thickness. Typical ray diagrams are shown in the inset of Fig. 1. The ray path involves both the deep seismic zone and the 400 km transition zone.

Figure 2 shows the seismograms of the Ryukyu earthquake of 7 January 1976 (24.4°N , 127.6°E , $h=58\text{ km}$; epicentral distance $\Delta=13.7^{\circ}$ at station DDR). The seismograms are presented in order of epicentral distances so that a vertically straight line gives the apparent velocity of 8 km s^{-1} although exact onsets are difficult to read because of small amplitude. The newly identified phase (X phase) is observed at epicentral distances from 12.5° to 14° and in this distance range the amplitude ratio of X to the first arrivals seems to increase with distance. The amplitude ratio is about 8 at station DDR. Although the X phase can be traced further up to $\Delta=14.5^{\circ}$ (station TSK), it is no longer a very prominent phase. The X arrivals are fitted by a straight line with the apparent velocity of 16.5 km s^{-1} . The fit is quite good. The value of 16.5 km s^{-1} is anomalously high and suggests that the X waves are steeply incident at the base of the crust beneath the network. We have observed the similar phase for other two Ryukyu earthquakes of 26 October 1972 (27.4°N , 128.5°E , $h=47\text{ km}$; $\Delta=12.5^{\circ}$ at DDR) and of 17 March 1974 (27.3°N , 128.0°E , $h=74\text{ km}$; $\Delta=12.9^{\circ}$ at DDR). The epicentral distance to station DDR is different among the three events but the maximum amplitude of the X phase always occurs around DDR. This feature is not explained by a horizontally layered Earth model.

On the basis of our interpretation for the X phase (Fig. 1, inset), we made a two dimensional ray tracing calculation. The two-dimensional treatment is warranted by a geographical relation among the epicentres, the network and the strike of the deep seismic zone (Fig. 1). The calculation involves the assumptions that the seismic zone is 4–12% faster than its continental side of the mantle which is modelled by the Kanamori¹ P-velocity model and that the boundary intersects the Earth's surface at a dip angle of 40° . The focus is placed at the surface in the distance range of $\Delta=14.5\text{--}17.5^{\circ}$ from the above intersection. A velocity contrast of more than 12% is not considered in

view of various lines of seismological evidence². Figure 3 shows the theoretical reduced travel time curves and the corresponding ray diagrams for $\Delta_0=15.5^{\circ}$ that can be compared to Fig. 2 after correction for the focal depth. The A, B and C branches are the direct results of the Kanamori model. The X and Y branches are produced by post-critical reflection from the inclined boundary of the velocity contrast. Important features are the following. (1) Amplitude: each branch is dotted according to equal interval of

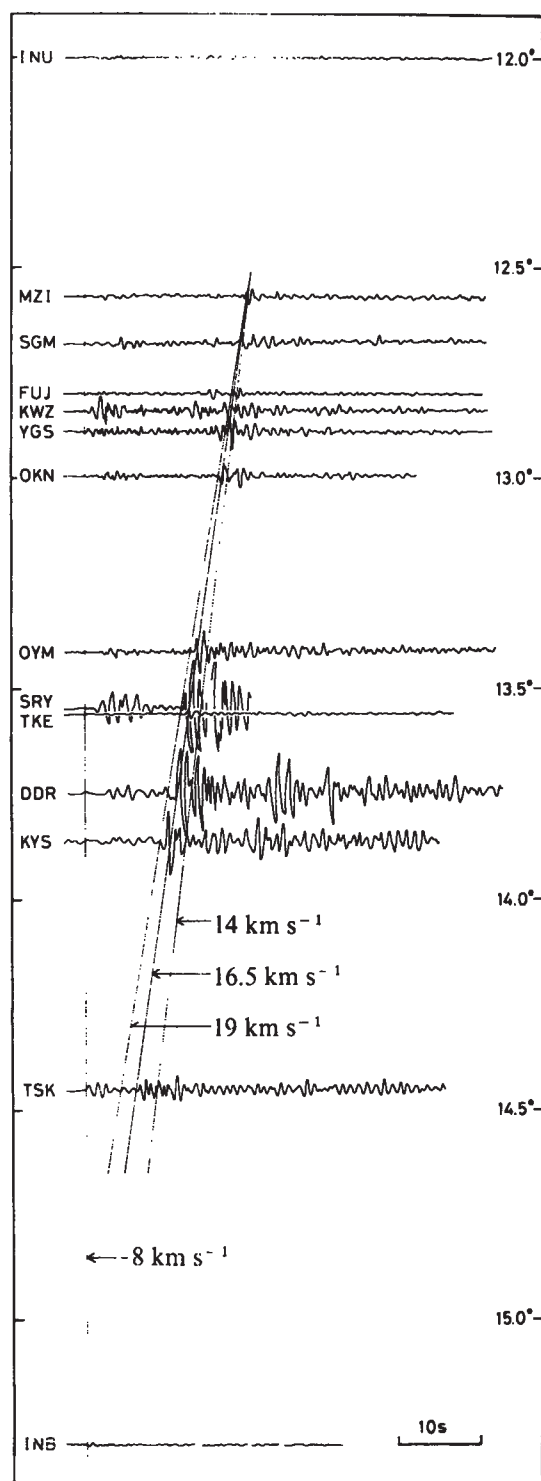


Fig. 2 Seismograms for the earthquake of 7 January 1976; at 18 h 32 min 10.2 s. (24.4°N , 127.6°E , $h=58\text{ km}$, 5.3 mb.) The straight line with the apparent velocity of 8 km s^{-1} marks roughly the first arrivals. The X arrivals are fitted by a straight line with the apparent velocity of 16.5 km s^{-1} . Two other lines are drawn for reference.

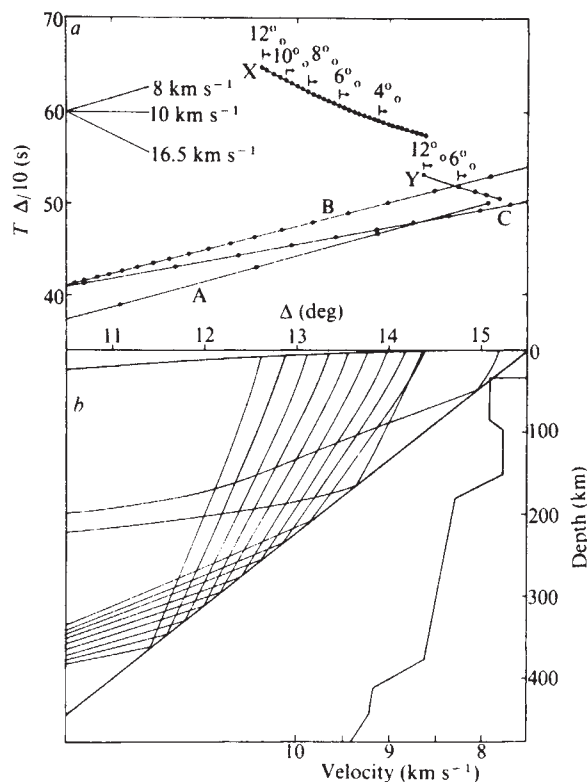


Fig. 3 *a*, Reduced travel time curves for the Kanamori¹ velocity model (A, B and C branches). Introduction of a deep seismic zone modelled by dipping velocity contrast produces additionally the X and Y branches. A larger velocity contrast (shown by the number) extends these branches back to shorter distances. Each branch is dotted so that the density of the dots can give a measure of the relative amplitude of that branch. *b*, Result of the ray tracing calculation for the Kanamori¹ velocity model with dipping velocity contrast. Only the rays belonging to the X and Y branches are shown, for simplicity.

slowness so that the density of the dots can give a measure of the amplitude of that arrival. The X branch is associated in Fig. 3 with an area dense with dots because of the strong ray convergence due to the 400-km transition, whereas other branches are all relatively weak. This result is consistent with the observation. (2) Apparent velocity: the apparent velocities of the X and Y branches are primarily controlled by the dip angle of the deep seismic zone and are insensitive to the detailed structure of the upper mantle. A remarkable agreement between the observed and calculated apparent velocities of the X phase suggests that our interpretation for the X phase is basically correct. (3) Distance: the distance ranges of the X and Y branches depend on the velocity contrast across the boundary. A larger velocity contrast produces the X and Y arrivals extending further back to shorter distances (Fig. 3). The observed distance range can be explained by a velocity contrast of 8–12%. (4) Relative arrival time: the observed time difference between the first and X arrivals is slightly smaller than the calculated time difference between the A and X arrivals and closer to the difference between the B and X. The discrepancy, however, is possibly within the uncertainty of the accuracy of the model. In the related distance range the exact locations of the A to C branches critically depend on the detailed structure of the uppermost mantle which varies from region to region. (5) Δ_0 dependence: the intensity and the distance range of the X branch depend on the value of Δ_0 . A Δ_0 value produces the stronger X arrivals at smaller epicentral distances over a narrower distance range. Only a very limited range of Δ_0 (15–16.5°) can produce the strong X arrivals in a distance range wide enough to identify the phase from other phases. This explains why the X phase

has been so far overlooked at the Dodaira Micro-Earthquake Observatory. (6) Effect of the 400 km transition: we made a similar calculation using the Jeffreys model which does not have the 400-km transition. In this case only one reflection branch, similar to the Y branch shown in Fig. 3, appeared. Its relative intensity and the distance range are grossly different from those of the observed later phase. This result indicates that the observed phase is a dual effect of the deep seismic zone and the 400-km transition.

We have so far assumed the boundary to be a first order discontinuity. Although this assumption has led to no inconsistency, the observations may also be explained in terms of a finite width of the boundary. To check this possibility, we placed a second order discontinuity at the boundary which causes strong refraction of the ray. The thickness of the boundary was varied from 0 to 30 km. The ray tracing calculation showed that as the thickness increases, the observable distance range of the X phase is rapidly shortened. Since the observable range can be widened by an increase of the velocity contrast, there is some trade-off between the thickness of the boundary and the velocity contrast across it. To explain the observation, the thickness must be less than 10 km for the velocity contrast of 10% and less than 20 km for the contrast of 12%. This result suggests that the boundary is so sharp that the thickness is within a wavelength of short-period (~ 1 s) P waves. Such sharpness has been suggested for other deep seismic zones from studies³ of ScSp which is a converted ScS phase arising from the dipping boundary of the velocity contrast.

We have thus demonstrated that the newly identified phase can be explained by post-critical reflection from an upper mantle discontinuity associated with a deep seismic zone. This phase is potentially very useful to elucidate the fine structure of a deep seismic zone. Based on the available data and the Kanamori¹ velocity model, we tentatively conclude that the Izu-Bonin deep seismic zone is 8–12% faster than its continental side of the mantle at their boundary at depths between 200 and 350 km, and that the transition thickness must be less than 10–20 km.

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Function of rodlets on the surface of fungal spores

THE surface of many fungal spores, including those of some members of the ascomycetes, basidiomycetes and deuteromycetes, is covered by a thin layer of regularly arranged 'rodlets'^{1–3}. The limited data available on their chemical nature suggest that these rodlets are composed largely of protein with some polysaccharide^{4–6}. Functions suggested for the rodlet layer have included water repellency⁴ and protection against dehydration⁷ but little experimental evidence has been available. We report here such evidence, based on a study of a mutant of *Neurospora crassa* that differs from the wild type in that its asexual spores (conidia) are both easily wetted and do not disperse readily in air currents. The conidia of the mutant, unlike those of the

wild type, lack rodlets on their surface.

To examine the conidial surface in a condition as close as possible to its natural state, we made surface replicas of unfixed, unfrozen, conidia that had been scattered dry on to the surface of freshly cleaved mica. Wild-type conidia were found to be covered entirely with rodlets (Fig. 1a and c), resembling in size and general appearance those described for other fungi¹⁻³. In contrast, conidia of the easily-wettable (*eas*) mutant had no rodlets and the wall appeared smooth (Fig. 1b and d). The presence of rodlets in wild type and their absence in the *eas* mutant was confirmed using freeze-fracture and deep-etch techniques.

The mutant differs from wild type by a single gene that has been tentatively mapped to linkage group II (ref. 6). It is indistinguishable in gross morphology from the wild-type (*eas*⁺) strain, but can be distinguished readily by two physiological criteria⁶. First, wild-type conidia are water repellent, whereas those of the *eas* mutant are readily wetted. If a drop of water is placed on the mass of aerial hyphae and conidia which forms at the top of agar slant cultures, it is not absorbed by the wild-type strain but remains intact on

top, whereas with the *eas* mutant it is readily absorbed. Second, the mutant differs in the aerial dispersibility of its conidia. In the laboratory, wild-type strains have a notorious reputation because of the ready manner in which conidia are released into the air during transfer of cultures. Conidia of the *eas* mutant, however, are not readily released even when a mass of aerial hyphae and conidia are shaken vigorously. Since the *eas* mutant conidia disperse readily in water, this lack of aerial dispersibility cannot be attributed, as in conidial separation (*csp*) mutants⁷, to a failure of individual conidia to separate from their neighbours in the proconidial chains. Rather, the chains break up, but the conidia so released stick to each other, and to the subtending aerial hyphae, forming large clumps.

Our finding that the *eas* mutant lacks rodlets provides evidence that the presence of rodlets on wild type *N. crassa* conidia renders them water repellent and easily dispersed by air currents. As spores of other species which have rodlets are frequently water repellent and dispersed by air, rodlets probably serve the same function in these species.

The water repellent property of wild-type conidia cannot

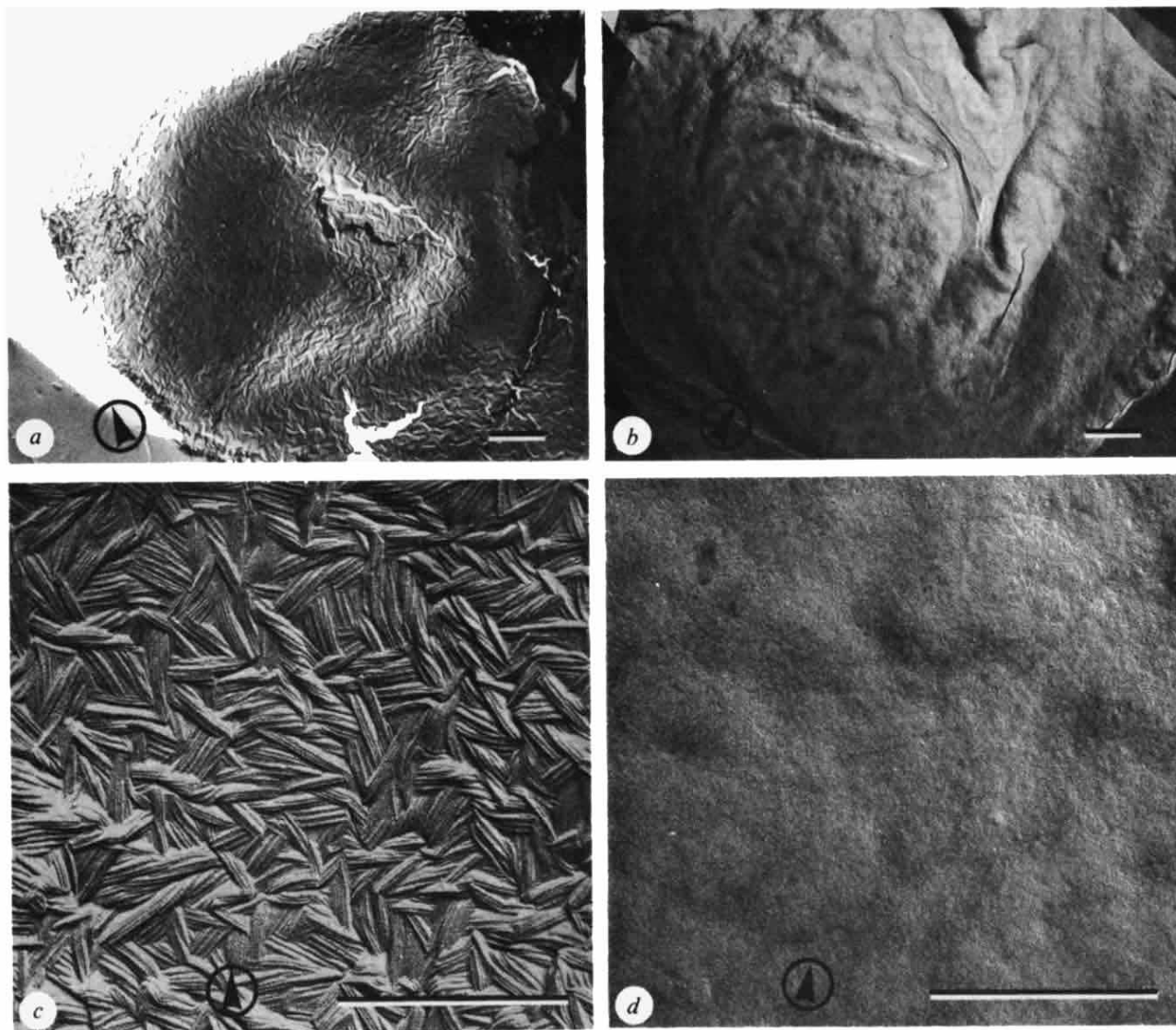


Fig. 1 Replicas of the surface of conidia of *N. crassa* wild type (a and c) and the *eas* mutant (b and d). Conidia were prepared by growing the strains on slopes of Vogel's medium N supplemented with sucrose (20g l⁻¹) and agar (15g l⁻¹) for 7 d at 25 °C. A sample of the mass of aerial hyphae and conidia (macroconidia) was removed and held above sheets of freshly cleaved mica. Gentle tapping was sufficient to dislodge conidia and hyphal fragments of wild type, but with the *eas* mutant the mass needed to be teased apart and tapped more vigorously. The sheets of mica were placed in a vacuum evaporator, shadowed at 45° with carbon/platinum, and a backing layer of carbon was then evaporated on at 90°. The replica was floated off on to water, the conidial material removed by treatment with chromic acid and a commercial hypochlorite bleach, and examined with a JEOL JEM-100B electron microscope. The direction of carbon/platinum shadowing is indicated by an encircled arrowhead. Bars represent 0.5 µm.

readily be attributed to any of the known chemical components of the rodlet layer but can be reasonably ascribed to the surface conformation⁴. The non-clumping of wild-type conidia, and their consequent ready dispersibility, can be accounted for by the dryness and unevenness of this surface.

The *eas* strain (UCLA191, FGSC 2961) was obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, California. The wild-type strain, St Lawrence's STA (74A), was obtained from J. R. S. Fincham.

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Scaling of sexual dimorphism in body size and breeding system in primates

RENSCH^{1,2} showed, more than 20 years ago, that sexual dimorphism in body size tends to increase with increasing body size in various arthropod and avian taxa. Recently, the same positive relationship has been suggested for mammals in general³, and primates in particular⁴. Based on their findings on primates, Clutton-Brock *et al.*⁴ discussed several possible functional explanations for this relationship, but considered none of them wholly satisfactory. In particular, they rejected the hypothesis that positive allometry in sexual dimorphism in weight may be the product of an association of size and polygyny; they did not present statistical data to that effect, however. I argue here that, on the contrary, there is a strong relationship between polygyny and positive allometry for sexual dimorphism in body size. The evidence is based on an analysis of the relationship between the scaling of sexual dimorphism in body weight and the breeding system for 53 primate species, which in most cases coincide with those chosen by Clutton-Brock *et al.*⁴ for their study. The findings are incorporated into a multifactorial system on the evolution of sexual dimorphism in body size.

The body weight data used in this study are those of adult males and females. Each species is represented by male and female means of a total of at least 16 individuals, with approximately equal numbers of each sex. Although the data are taken from various sources and therefore not perfectly comparable, a major source of error usually found in body weight studies has been eliminated by disregarding that data from captive animals and using data only from wild-trapped and wild-shot animals⁵. Regressions of $\log_{10}$ (male weight) on $\log_{10}$ (female weight) were fitted by least squares and major axis techniques. As correlation coefficients in all instances are only slightly below 1.0 (Table 1) and thus the differences between least squares and major axis slope negligible, only values of the least squares slope were used for further computations and discussion. The regressions were tested for equality of slope with the isometric slope for geometric scaling, which in a weight-weight relationship is 1.0.

Table 1 shows that for combined polygynous and monogamous species male weight scales strongly at a power of female weight of 1.081. The slope is significantly different from 1.0 at $P < 0.01$. This suggests that sexual

dimorphism in body weight tends to increase exponentially with increasing body weight, which agrees with the findings of Clutton-Brock *et al.*⁴. However, a separation of the species on the basis of the breeding system yields different results (Table 1 and Fig. 1). While for 42 polygynous species male weight is positively related to female weight (the slope of 1.085 is significantly different from 1.0 at $P < 0.01$), for 11 monogamous species the slope of 1.002 shows equality with the isometric slope of 1.0. Furthermore, the y-intercept is practically zero. As a numerical example, a polygynous species in which adult females weigh 1,000 g would have adult males that weigh 1,211 g, while a monogamous species with the same female weight would have males that weigh 1,033 g. For females averaging 10,000 g, the predicted weight for males of a polygynous species would be 14,730 g, while male weight of a monogamous species would be 10,380 g. This suggests that while in polygynous species sexual dimorphism increases exponentially with increasing body weight, in monogamous species sexual dimorphism not only remains constant throughout the size range, but actually is minimal or lacking at any given body weight.

The observation that in polygynous species sexual dimorphism in body weight tends to increase exponentially with increasing body weight may be modelled by a multifactorial system. I consider that the more highly polygynous a species is (the fewer males than females that contribute gametes to zygotes), the more intense is sexual (either intrasexual or epigamic⁶) selection and thus selection for larger male size. Increased male size in turn promotes polygyny. In an intricate feedback, evolution of larger size is interrelated with an increase in sexual dimorphism in morphological (for example, canine size⁷), physiological (sexual maturity³) and behavioural (social role differentiation⁷) features. This model is in accordance with a similar one developed on the basis of observation of grouse by Wiley⁸, who argues that the evolution of polygyny is inseparable from the evolution of large size.

This model applies particularly well to the large-sized cercopithecoid and pongid species. Arid-country, savannah, and forest baboons (*Theropithecus*, *Papio*, *Mandrillus*) and the gorilla (*Pan gorilla*) are the most strongly dimorphic primates. The model also applies to the orangutan (*Pongo pygmaeus*), although McKinnon⁹ claims that this animal is more dimorphic than could be expected from its present-day breeding system. To account for the discrepancy, he postulates the strong dimorphism to be a relict of earlier times when large males enjoyed more of a reproductive advantage. A species that at first sight is less dimorphic than could be expected from its body size is the chimpanzee (*Pan troglodytes*). However, the chimpanzee is much smaller than the gorilla, suggesting that phyletic size increase in the former was much smaller than in the latter after their divergence in the Late

Table 1 Regressions of $\log_{10}$ (male weight) on $\log_{10}$ (female weight) for primates on the basis of the breeding system

Breeding system	N	a	S _a	b	S _b	r ²	t
Combined polygynous and monogamous species	53	-0.182	0.086	1.081	0.022	0.979	3.66*
Polygynous species	42	-0.172	0.088	1.085	0.024	0.981	3.54*
Monogamous species	11	0.008	0.075	1.002	0.022	0.996	0.10

N, sample size (no. of species); a, y-intercept; S_a, standard error of a; b, least squares slope; S_b, standard error of b; r², squared correlation coefficient; t, t-value of difference of slope from isometric slope at 1.0.

*Significant difference at $P < 0.01$.

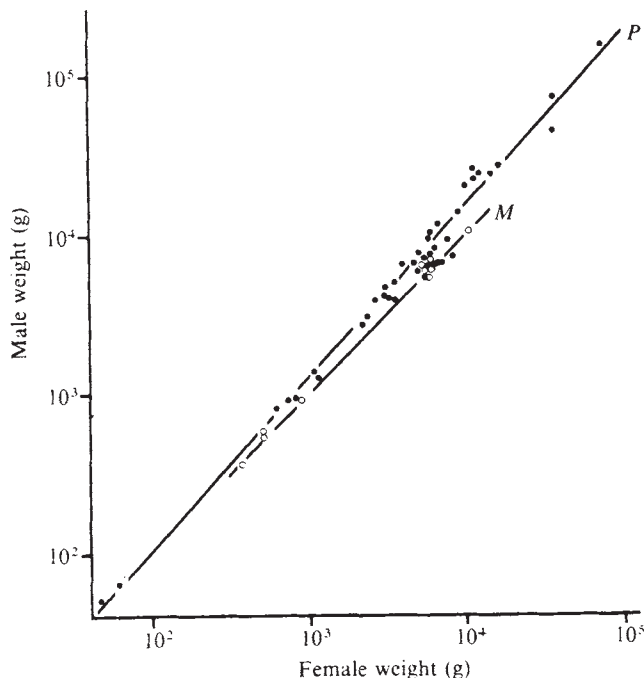


Fig. 1 Double-logarithmic representation of the relationship between adult male and female weights for 53 primate species (based on Table 1). The upper line (*P*) represents the regression for 42 polygynous species (designated by solid dots); the lower line (*M*) represents the regression for 11 monogamous species (designated by open dots).

Tertiary. The argument of a relatively small phyletic size increase in the chimpanzee is in accordance with the observation of relaxed sexual selection as a correlate of high male tolerance and promiscuity in this species⁷.

While there is high predictability from large body size to strong sexual dimorphism, for a few small- and medium-sized species, the predictability for the degree of sexual dimorphism is not as good. In the black-handed spider monkey (*Ateles geoffroyi*) and some colobine species (*Colobus verus*, *Presbytis aygula*, *P. melalophos*, *P. frontata*), sexual dimorphism is as minimal or lacking as in monogamous species. This clearly indicates that the intensity of sexual selection cannot account for all variation in sexual dimorphism. Species-specific selection pressures which tend to reduce male size and thus sexual dimorphism are varied and numerous (for example, abundance and distribution of food, predator avoidance, positional repertoire, bioenergetic constraint^{3,4,7}). There seems to be agreement, however, that for most primate species the intensity of sexual selection is the main factor determining the degree of sexual dimorphism^{7,10}.

The observation that for monogamous species sexual dimorphism is minimal or lacking and independent of body weight can be explained by the finding that for all species for which data are available, the intensity of sexual selection and the degree of behavioural dimorphism are similarly low, while male parental investment is similarly high. For example, the summarised intensity of paternal investment reflected by particular types of behaviour such as defence of territory, and defending, grooming, feeding, carrying and teaching the young is almost identical for small-sized species such as marmosets (*Callithrix*) and tamarins (*Saguinus*, *Leontopithecus*) to that for the largest-sized monogamous species, the siamang (*Hylobates syndactylus*)¹¹. It is also notable that monogamy, not only in primates but also in other mammals (such as carnivores¹¹) is almost entirely limited to small- and medium-sized species. This may be explained by the lowered

intensity of sexual selection, thus reducing selection for large male size, one consequence of which is a slowing in phyletic size increase. It may even be argued that phyletic dwarfism as seen in marmosets and tamarins^{12,13} is at least partially associated with monogamy in these primates. Because of lack of selection for larger male size, selection for a size decrease as an adaptive response to a secondary shift to a higher energy diet¹⁴ may have acted more strongly, and resulted in a phyletic size decrease.

Therefore, while polygyny promotes an increase in both sexual dimorphism in body size and body size *per se*, monogamy promotes monomorphism and tends to limit body size. Although this model has been developed based on primate species, and certainly needs further testing on other taxa, it provides new insights into the causes of scaling in sexual dimorphism in body size.

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Light-controlled production of spawning-inducing substance in jellyfish ovary

THE jellyfish *Spirocodon saltatrix*, a bell-shaped member of the hydromedusae between 5 and 8 cm in diameter, spawns soon after nightfall; placing the animal in the dark causes a copious release of mature gametes within approximately 2 h, regardless of the day–night relationship^{1–3}. Oocytes taken from the ovaries during the morning and early afternoon are immature and contain germinal vesicles whereas spawned eggs are always fully mature⁴. When isolated from the animals, the ovaries discharge eggs in the light 1–2 h later and the remaining oocytes inside the ovaries are small⁵. When these ovaries are placed in the light (about 8,000 lx at the surface of the water) they gradually increase in size, becoming fully grown after 15 h. We report here that the isolated ovaries discharge eggs 50–70 min after a 30-min exposure to the dark, during which period a spawning-inducing substance of low molecular weight is produced in the ovaries. The ovaries fail to produce this substance in continuous illumination.

Four ovaries from a single female were kept separately in seawater in the light for 15 h at 12 °C. Subsequently, two of them were kept illuminated and the other two were placed in the dark. A 30-min exposure to darkness was enough to induce spawning 80–100 min after the initiation of the dark treatment regardless of the photic conditions after treatment (Fig. 1).

We next examined the possibility that darkness-induced spawning in the isolated ovaries is mediated by a 'diffusible' chemical factor(s). The ovaries taken from 11 females were illuminated for 15 h, and then transferred, two from each individual, into a dish containing 20 ml of seawater and kept in the dark. The remaining 22 ovaries were trans-

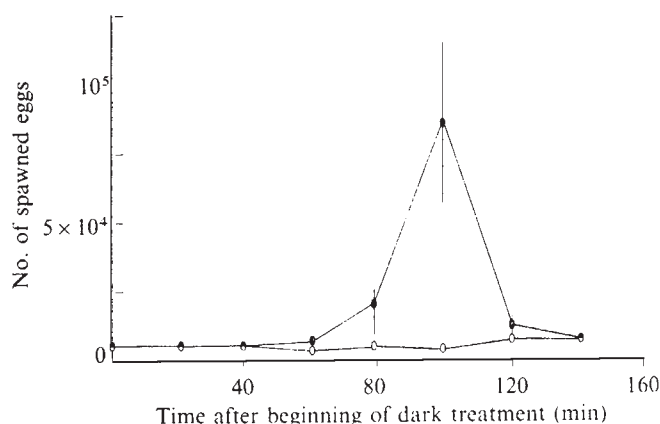


Fig. 1 Spawning of a *Spirocodon saltatrix* ovary induced by darkness. An ovary was placed in 50 ml of seawater and illuminated for 15 h at 12 °C, darkened for 30 min and returned to the light. The number of eggs released into the seawater was noted every 20 min after the beginning of the dark treatment. Mean values of six experiments are shown. ●, Experiment; ○, control without dark treatment. Vertical bars represent the s.e.m. values.

ferred into a dish containing 20 ml of seawater and placed in the light. Thirty minutes later, the ovaries were removed by filtration through two layers of cheesecloth. Filtrates were centrifuged separately at 3,000 r.p.m. for 20 min. The supernatant obtained from the dark-treated dish and that from the light-treated dish were designated ovarian medium-30 and medium-0, respectively. Bioassay showed that the ovaries treated with medium-30 and kept illuminated discharged 4.3 ± 1.9 (mean $\pm$ s.e.) times more eggs than controls (Table 1). On the other hand, medium-0 did not affect spawning in the light (Table 1). These results suggest that some active substance(s) is liberated from the ovary into the medium during 30 min in the dark.

Medium-120 (6 ml), which had contained 10 ovaries for 120 min in the dark, induced spawning (Table 1). Medium-120 diluted eight times with seawater induced spawning whereas four times diluted medium-30 did not affect spawning (Table 1). The spawning-inducing activity in medium-120 was completely removed by treatment with agarose-bound

trypsin (Miles) or agarose-bound *Streptomyces griseus* protease (Miles) for 2 h at 28 °C. Therefore some peptidyl linkages seem to be present in the active substance(s) and they may be essential for the biological activity. Medium-120 (4 ml) was applied to a column (1.7 $\times$ 40 cm) of Sephadex G-15 (Pharmacia), which was equilibrated with artificial seawater. The column was eluted with the seawater into 4-ml fractions at a flow rate of 12 ml h⁻¹. The spawning-inducing activity was detected in fractions 13 and 14. Blue dextran and cupric sulphate were also gel-filtered on the same column and eluted in fractions 10 and 18, respectively, suggesting that the molecular weight of the active substance is less than 1,500. These results suggest that the ovaries produce a peptide of low molecular weight during the dark period and that it triggers oocyte maturation and subsequent spawning.

To examine whether tissues other than ovaries produce a spawning-inducing substance, medium-30 was prepared from 22 ml each of seawater which had contained the manubria, tentacles and testes of ten individuals for 30 min in the dark. Medium-30 from the manubria inhibited spawning, that from the tentacles had little effect on spawning and that from the testes induced spawning (Table 1). Therefore the spawning-inducing activity is present only in diffusates from gonads and the water canals that run through the manubria, gonads and tentacles make no contribution.

In many hydrozoans, such as *Hydractinia epiconcha*¹, *Eutima mira*² and *Gonionemus murbachii*³, darkness induces spawning. Molecular mechanisms involved in the process of spawning are poorly understood. To the best of our knowledge, our results are the first demonstration that light-controlled spawning in coelenterates can be mediated by a diffusible substance⁴.

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Table 1 Effect of diffusates of tissues on spawning of isolated *Spirocodon saltatrix* ovaries

Tissue	Period of dark treatment (min)	Concentration (mg dried tissue per ml)	Spawning index*
Ovary	0	56	1.2 $\pm$ 0.5
	30	56	4.3 $\pm$ 1.9
	30	14†	0.9 $\pm$ 0.1
	120	80	5.1 $\pm$ 2.4
	120	10†	1.7 $\pm$ 0.3
Manubrium	30	32	0.4 $\pm$ 0.2
Tentacle	30	109	0.8 $\pm$ 0.3
Testis	30	87	8.5 $\pm$ 4.2
	30	22†	1.4 $\pm$ 0.1

Diffusates were prepared by placing fresh tissues in seawater at the concentrations indicated in the third column in the dark for the periods indicated in the second column and subsequently removing the tissues. To determine the effects of the diffusates on spawning, two ovaries from a single female were placed in seawater at 12 °C in the light for more than 15 h. One ovary was placed in a dish containing 3 ml of a sample solution and another ovary was placed in a dish containing 3 ml of seawater, and they were kept in the light. The ovaries were removed from the dishes 150 min after the initiation of sample application, two 10- μ l samples of the egg suspensions were sucked up and numbers of the eggs were counted.

*Expressed as the ratio of the number of the shed eggs in the sample solution to that in the seawater, mean $\pm$ s.e.m. control.

†Diffusates were diluted with seawater and bioassayed.

Attenuation of positional signalling in the chick limb by high doses of γ -radiation

THE spatial pattern of cellular differentiation in the chick limb can be viewed in terms of positional information. For the antero-posterior axis of the wing, positional information seems to be specified by reference to a group of cells at the posterior margin of the limb bud—the zone of polarising activity (ZPA)^{1–3}. By grafting an additional ZPA to different positions along the antero-posterior axis, we found that structures form according to their distance from the nearest ZPA. For instance, digit 4 forms nearest to the ZPA, then digit 3, then digit 2. We suggested that cells might measure distance by means of a diffusible morphogen produced by the ZPA, the concentration of which falls as distance from the ZPA increases. The responding cells are able to interpret the concentration of the morphogen, and so behave according to their position. If it is the concentration of morphogen that specifies which digit shall form,

then lowering the concentration of morphogen adjacent to the ZPA should result in digit 3 being formed next to the ZPA instead of digit 4. If the concentration is lowered still further, digit 2, and finally no additional digit should form next to the ZPA. We have obtained such an attenuation of positional signalling by treating the ZPA with increasing doses of γ radiation.

Quail embryos at stages 21–23 were irradiated *in ovo* in a Vickers-Armstrong Mk IV Hotspot irradiation unit at a dose rate of $1.6 \text{ krad min}^{-1}$ as measured by ferrous sulphate dosimetry. The limb buds were removed from the embryos and the ZPAs grafted to sites prepared in the anterior margins of embryonic chick wings (stages 18–21) usually opposite somite 16 and occasionally opposite the junction between somites 16 and 17. Grafted to these positions, the pattern of digits produced by a non-irradiated ZPA is usually 432234, 43234 or 4334 (ref. 2); that is, an additional digit 4 is usually formed posterior to the graft. All the grafts were carried out within 1.5 h of completion of irradiation, and they were examined the following day to ensure that they had taken. The host embryos were incubated for a further 6 or 7 d; the wings were then fixed and the pattern of digits examined in whole mounts.

The results of the grafts are presented in Table 1. For simplicity, only the additional digit next to the graft is indicated. It is clear that as the dose of radiation increases from 5 to 96 krad the capacity to form digit 4, then digit 3 and finally digit 2, decreases (Fig. 1). We interpret this as being due to a progressive attenuation of the signal from the ZPA. As controls, at least three grafts of tissue from the anterior margin of the wing bud were made at each dose of radiation, and in no case were extra digits produced. A similar attenuation of positional signalling was obtained with chick ZPA, but at lower doses of radiation.

These results are consistent with the hypothesis that there is a gradient of positional information along the antero-posterior axis of the chick wing. They cannot be explained by a polar coordinate model⁴, and argue against any model based on sequential induction of digits, wherein specification of digit 4 is followed by specification of digit 3, and then specification of digit 2.

It is remarkable that such heavily irradiated ZPA cells are capable of even attenuated positional signalling. A similar ability is seen in the development of insects, where *Drosophila* imaginal disks can still stimulate intercalary regeneration after doses of radiation up to 100 krad (ref. 5), and in the coelenterate hydra, where positional signalling will occur after 25 krad of γ irradiation⁶. In both these cases also, there is evidence that irradiation produces an attenuation of positional signalling. We do not know how irradiation attenuates positional signalling, but there are three targets that could be affected. These are the signal itself, the production of the signal, and the channel through which the signal passes. At present we cannot distinguish between these possibilities.

It is clear, however, that cell division is not required for positional signalling. Irradiated leg ZPAs never produced toes in the host wing, whereas three out of three non-irradiated leg ZPAs did (see also ref. 3). Furthermore, histological sections of a limb 24 h after a graft of a ZPA irradiated with 24 krad of γ radiation showed no normal

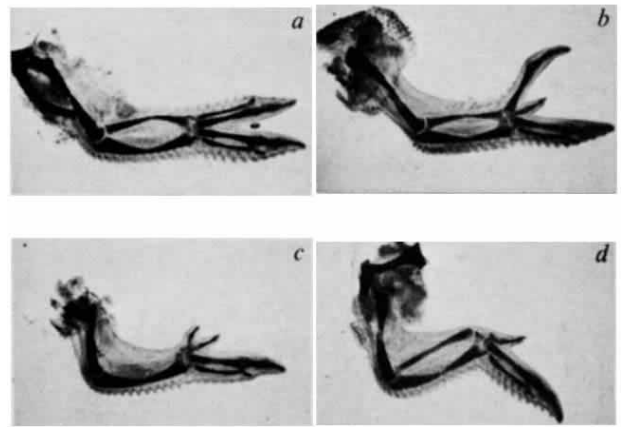


Fig. 1 A series of dorsal views of whole mounts of right wings showing the pattern of digits formed as the dose of radiation imparted to the ZPA increases. The digits, from anterior to posterior, and the dose of radiation, are: a, 43234, 12 krad; b, 3234, 24 krad; c, 2234, 64 krad; d, 234, 96 krad.

mitotic figures in the graft. These non-dividing ZPA cells remain in a proximal position as the limb grows out, and this has enabled us to show that the signalling property of the irradiated cells is not passed on to adjacent cells. This indicates that a brief exposure to the ZPA is sufficient to specify positional information, and explains why a normal limb can develop when the ZPA is removed^{2,7,8}.

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An extravascular site of development of *Trypanosoma congolense*

TSETSE-TRANSMITTED African pathogenic trypanosomes are thought to be divisible into two groups because of their distribution in the mammalian host and the characteristic lesions they produce during infection¹. *Trypanosoma brucei* and related subspecies have a wide distribution in the body, parasitising the intercellular fluids, connective tissue and parenchymatous tissues, and the fluids of body cavities^{2–4}, whereas *T. vivax* and *T. congolense* are considered to be strictly plasma parasites, confined to the circulatory system^{5,6}. It has been shown, however, that local skin reactions develop at the sites of bites of tsetse flies infected with *T. congolense*, and fluid expressed from reaction sites contains trypanosomes several days before they can be detected in the peripheral blood^{7–9}. We have recently examined the development of *T. congolense* in local reactions and give evidence here from light and electron microscopy that the trypanosomes develop in connective tissue. The multiplication and persistence of trypanosomes

Table 1 The additional digit formed next to the ZPA as the dose of radiation increases

Dose of radiation (krad)	4	3	2	None
Control	11	1	—	1
5	6	2	—	—
10	5	2	—	—
24	3	10	1	1
64	—	4	4	6
96	—	1	2	6

at the site of infection have important implications in relation to immunogenesis and might also play a part in the pathogenesis of the ensuing disease and the efficiency of chemotherapy.

Glossina morsitans, infected experimentally with *T. congolense*, were fed on the backs of New Zealand White rabbits. Local reactions appeared 5-6 d later and developed into well-defined nodules which reached 25 mm in diameter by day 9 and persisted until day 15. Trypanosomes were

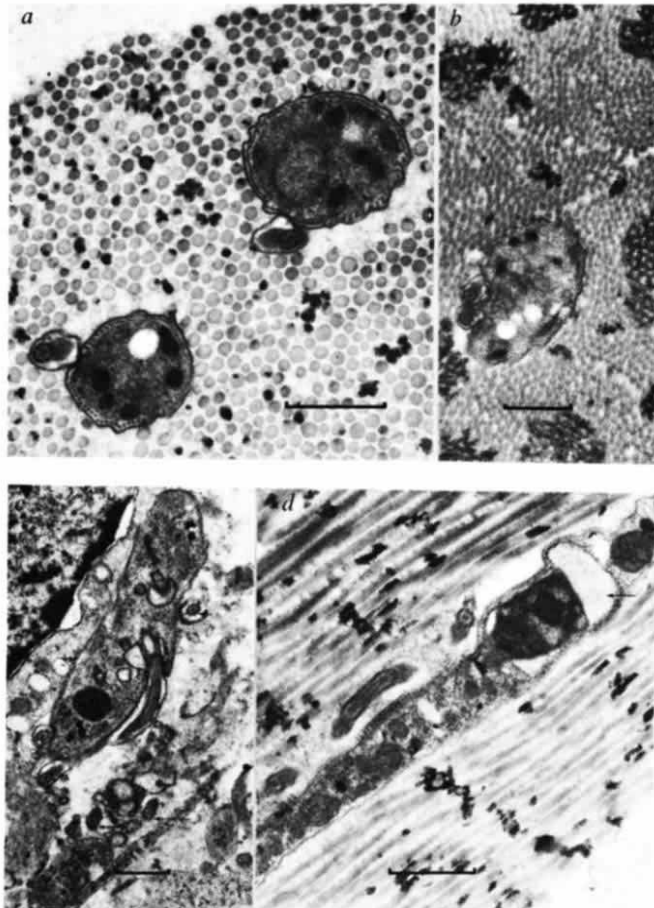


Fig. 1 Electron micrographs of trypanosomes in rabbit tissues taken from local skin reactions at sites of infection with *Trypanosoma congolense* transmitted by *Glossina morsitans*. Newly emerged *Glossina morsitans* from the Tsetse Research Laboratory, Langford, Bristol, were fed through chicken skin membranes on pools of mouse blood containing *T. congolense* derived from stabilate TREU 1290, and then maintained on rabbits for 28 d. Infective flies were identified by finding metacyclic trypanosomes in smears of tsetse saliva on glass slides, or by feeding on indicator rabbits. Infective flies were fed on the shaven backs of New Zealand White rabbits from which reactions were excised after killing at 7, 9, 11 and 19 d. For light microscopy, thin transverse slices of skin through the lesions were fixed in Zenker-formol solution for 12 h, dehydrated and then embedded in paraffin wax: 6-µm sections were cut and stained by haematoxylin and eosin and Giemsa-colophonium. For electron microscopy, small blocks of tissue approximately 1 mm³ were fixed in 2.25% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 for 2 h, rinsed in cacodylate buffer for 15 min and then postfixed in 1% buffered osmium tetroxide for 60 min. The blocks were washed in buffer, dehydrated, and embedded in araldite. Ultra-thin sections were cut with a glass knife and stained with lead citrate and uranyl acetate. *a*, Two trypanosomes in cross section surrounded by collagen microfibrils in 7 d reaction; *b*, trypanosome with two flagella, indicating early stage of division in collagen from 7 d reaction; *c*, section through flagellar pocket region of dividing trypanosome lying outside collagen microfibrils in 11 d reaction. Note also evidence of concurrent extracellular trypanosome destruction indicated by sections of isolated flagella lacking membranes (arrowed); *d*, longitudinal section through collagen from an 11 d reaction showing trypanosome undergoing lysis as indicated by swollen nuclear envelope (arrowed). Bars represent 1 µm.

found in serous fluid expressed from 7 d reactions, and parasites were present at the reaction site for at least 19 d after infection. They were first seen in the peripheral blood on day 14-19.

Microscopically, marked infiltration of the dermis by mononuclear cells was apparent from day 7, and by day 9 degenerative changes in the collagen were detected. Many trypanosomes were seen in bundles of collagen in the dermis in 7, 9 and 11 d reactions, mainly orientated longitudinally along the line of the collagen fibres. Electron microscopy showed trypanosomes surrounded by microfibrils of collagen (Fig. 1*a*). The collagen also contained dividing trypanosomes (Fig. 1*b*), indicating that the organisms were viable and not degenerating after failing to enter the vascular system. Trypanosomes were also seen outside the collagen bundles in preparations of 7, 9 and 11 d reactions, and in small vessels, considered to be lymphatics, after 7 and 9 d. In 11 d reactions, electron microscopy showed isolated, extracellular trypanosome flagella (Fig. 1*c*) and degenerating parasites (Fig. 1*d*).

It is thus erroneous to regard *T. congolense* as a parasite confined to the vascular system, particularly as trypanosomes have also been seen in collagen from skin reactions from calves infected with tsetse-transmitted stocks of this parasite. The demonstration of trypanosomes in connective tissues supports suggestions that tissue foci of parasites might contribute to a secondary stage of disease caused by *T. congolense*^{10,11}. The release of phospholipase A from *T. congolense* and the resultant local accumulation of free fatty acids, which have extensive effects on the endothelium of small blood vessels^{12,13}, might also have equally important effects on connective tissue. In addition, the long-established association of *T.b. brucei* with collagen¹⁴ has recently been used to considerable advantage in the development of a method for its cultivation with fibroblast-like cells¹⁵, and the present observations suggest that such cells might also effectively support the growth of *T. congolense*.

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Induction of antibodies against carcinogenic polycyclic aromatic hydrocarbons

MANY carcinogens can elicit antibodies when linked to macromolecular carriers¹⁻³. If antibodies against an entire class of carcinogens could be elicited by a non-carcinogenic analogue, and proved capable of neutralising the biological effects of the carcinogens, human immunisation might be of practical value. Mucosal (predominantly IgA) antibodies, capable of facilitating the excretion of an antigen before

its absorption into the body, might be particularly useful in this^{6,7}. Here we provide evidence that: an analogue of the polycyclic aromatic hydrocarbon (PAH) carcinogen dimethylbenzanthracene (DMBA), substituted with a fluorine moiety to abrogate carcinogenicity, can induce antibodies which bind a variety of PAH carcinogens; that these antibodies can protect tissue culture cells against one of the prominent *in vitro* biological effects of such carcinogens— toxicity; and that gastrointestinal mucosal immunisation against PAH carcinogens is feasible, and possibly represents a means of achieving *in vivo* protection by enhancing carcinogen excretion.

The DMBA analogue, 5-fluoro-12-methylbenzanthryl-7-acetic acid (FMBAAA) was used on the basis of evidence that fluorination or other substitutions at position 5 in DMBA and related compounds renders these compounds noncarcinogenic^{8,9}. In the present studies, FMBAAA (1.3 mg subcutaneously in 0.1 ml triolein) proved non-toxic and has induced no tumours after 16 months in 30 C57Bl/6J male mice. Doses of 2, 5, 10, 50 and 250 µg were non-mutagenic in the bacterial mutagenesis assay of Ames *et al.* using the *Salmonella* tester strain TA-100 and Aroclor-induced 'S9' fractions of rat liver homogenates¹⁰. In contrast, 1 mg of DMBA induced tumours in 16 of 30 mice, and was mutagenic in the Ames assay, yielding 54

Table 1. Binding of polycyclic hydrocarbons by guinea pig sera

Compound	Guinea pig no.	Specific binding (ng per ml antiserum)	Mean value
FMBAAA	1	234	135
	2	54	
	2	62*	
	3	112	
DMBA	1	49	97
	2	133	
	3	110	
BaP	2	95	95
BrsP	2	108	108
Cholic acid	2	-2†	-2†

FMBAAA was synthesised by the method of Bergmann and Blum¹⁷. Fifty mg was conjugated to 270 mg BSA by first forming a mixed anhydride of FMBAAA isobutylchloroformate¹⁸; the material was then mixed with BSA to allow amide linkages to form by way of the free amino groups of the protein. Like FMBAAA, the conjugate exhibited no toxicity or carcinogenicity in mice. For immunisation, three 350–400 g Hartley female guinea pigs were each injected intramuscularly with 0.2 mg of the conjugate emulsified in complete Freund's adjuvant and distributed among the four legs. Booster injections were given 2.5 and 6 weeks later, and the animals were bled 8 d after the last injection. Binding of ¹⁴C-DMBA and ³H- forms of the other compounds was measured by the use of a staphylococcal protein A method which uses this surface protein of *Staphylococcus aureus* as a solid phase adsorbent for IgG antibody molecules¹⁹. Duplicate to quadruplicate 0.2 ml quantities of antiserum, diluted 1/4 in phosphate-buffered saline (PBS) were mixed with 0.2 ml of radiolabelled hydrocarbon (diluted to 0.1 µg ml⁻¹ in 1:1 dioxane-water), followed 30 min later by addition of 0.4 ml of a 10% ethanol- or formalin-fixed *S. aureus* suspension in PBS. After an additional 2 h, the suspensions were washed twice by centrifugation in 20:1 PBS-dioxane and once by centrifugation in PBS, and were dissolved in NCS solubiliser (Amersham-Searle) for measurement of radioactivity by liquid scintillation spectrometry. Controls included the use of sera from three non-immunised guinea pigs. PAH binding with the non-immune sera was low (<30 ng ml⁻¹ serum) except in the case of BrsP (71 ng ml⁻¹); these values were subtracted from values obtained with immune sera to determine specific binding. The low values of nonspecific binding are comparable with those reported by others using *S. aureus*¹⁹.

*Results obtained when a 1/8 dilution of antiserum was used to determine binding. In aggregate, the results from both dilutions are significantly different from control values ($P < 0.05$ by student's *t* test).

†The negative value signifies less binding than that obtained with normal serum.

Table 2 Effect of anti-PAH antibodies on DMBA cytotoxicity

Treatment	% Cytotoxicity*	
	DMBA µg per well: 0.01	0.05
PBS	44.7 ± 5.2 (17)	42.4 ± 3.1 (14)
Normal guinea pig globulin	21.7 ± 4.5 (16)	37.9 ± 5.3 (14)
Anti-PAH globulin	0.6 ± 7.5 (13)	13.8 ± 13.6 (9)

Hamster embryo cells (2×10^4) were plated in plastic wells (Linbro) containing 0.2 ml Dulbecco's medium with 10% foetal bovine serum. After 18 h at 37 °C, the wells were aspirated, and the medium was replaced with either 0.05 ml PBS, or PBS containing 22 mg ml⁻¹ of globulin fractions obtained by ammonium sulphate precipitation of guinea pig antisera (Table 1) or normal guinea pig sera. An equal volume of medium containing 0, 0.01, or 0.05 µg DMBA (in dimethylsulphoxide at a final concentration of 1%) was then added. After incubation at 37 °C for 24 h, the cells were removed by trypsinisation, and were counted in a haemocytometer. The results, representing combined experiments with three normal and two immune sera, are expressed as mean ± standard error. The number of cultures in each treatment group is shown in parentheses. Cytotoxicity in cultures treated with immune globulin was significantly less than in cultures treated with normal globulin ($P < 0.02$ by student's *t* test for 0.01 µg DMBA and $P < 0.05$ for 0.05 µg DMBA) or with PBS alone ($P < 0.01$ for both DMBA levels).

*Calculated as:

$100 \times (\text{cell no. in cultures without DMBA} - \text{cell no. in DMBA-treated cultures}) \div (\text{cell no. in cultures without DMBA})$
The value obtained does not indicate how much of the DMBA effect is cytostatic and how much is cytotoxic; these two possibilities cannot be distinguished by this assay and are both encompassed by the term 'cytotoxicity'.

revertant colonies per plate at a dose of 2 µg, 640 at 10 µg and 1,534 at 50 µg, which is comparable with the results reported by Ames *et al.*¹⁰.

Table 1 shows that guinea pigs immunised with FMBAAA conjugated to bovine serum albumin (BSA) yielded antisera capable of binding the hapten used for immunisation, FMBAAA, and also the related PAH carcinogen, DMBA. One serum (serum 2) was also tested for its ability to bind two additional PAH carcinogens, benzo(a)pyrene (BaP), and benzo(rst)pentaphene (BrsP); in each case, binding was demonstrated. In contrast, no binding was observed with the non-aromatic polycyclic compound, cholic acid. Serum 2 was also analysed in other ways for confirmatory evidence of specificity. When 0.5 mg of rabbit IgG directed against guinea pig γ-globulins (Cappel Laboratories) was used instead of *Staphylococcus aureus* to precipitate antibody-FMBAAA complexes, similar specific binding values were obtained (70 ± 17 for duplicate samples). A 1/4 dilution of serum 2 produced a strong precipitin line when tested in an agar gel diffusion plate against the FMBAAA-BSA conjugate (1 mg ml⁻¹) used for immunisation. No line was observed against native BSA, suggesting that the BSA structure of the conjugate had been significantly altered by conjugation. Inhibition studies were done in triplicate by carrying out the ³H-FMBAAA binding assays in the presence of a tenfold excess by weight of non-radioactive FMBAAA, DMBA, or BaP. Reductions in specific ³H-FMBAAA binding of 82 ± 4%, 84 ± 7%, and 77 ± 9% respectively (mean ± standard error) were observed. In contrast, BSA failed to inhibit binding (<4% reduction), suggesting a specific inhibitory effect of the PAHs.

To determine whether antibodies from the above antisera could neutralise biological effects of PAH carcinogens, globulin fractions from these sera were tested in an *in vitro* cytotoxicity assay using hamster embryo cells¹¹, an assay chosen because (unlike *in vitro* transformation assays) it proved to be a reproducible way of measuring sensitivity to nanogram amounts of PAHs. The results of these protection experiments (Table 2) show that the antisera completely abrogated the toxicity of 0.01 µg DMBA, and greatly re-

duced the toxicity of 0.05 μg DMBA. Normal globulin also reduced DMBA toxicity, but to a much smaller extent. Whether the effect of normal globulin represented DMBA binding or a nonspecific enhancement of viability of DMBA-injured cells is unknown.

To determine whether immunisation could affect the fate of an ingested PAH carcinogen *in vivo*, 10-week old male A/J mice were immunised intraperitoneally (i.p.) with the FMBAAA-BSA conjugate emulsified in two volumes of complete Freund's adjuvant (0.1 ml of emulsion containing 0.2 mg antigen). Two weeks later, the mice received a booster i.p. immunisation without adjuvant. Two and four weeks after this, the mice were given 0.3 mg antigen (without adjuvant) by gastric intubation. ^3H -BaP was then used *in vitro* and *in vivo* to study gastrointestinal immunity. As shown in Table 3, the capacity of faecal γ -globulin to bind BaP *in vitro* was significantly higher in mice immunised with FMBAAA-BSA than in unimmunised mice, and the ability of such mice to excrete fed ^3H -BaP as γ -globulin-bound complexes was significantly higher in comparison with both unimmunised and BSA-immunised controls. Total (bound plus free) ^3H -BaP excretion was also slightly higher, and liver accumulation of ^3H -BaP slightly lower in FMBAAA-BSA mice (data not shown), but these differences were not significant. Larger differences might have been observed if the dose of ^3H -BaP administered had not so greatly exceeded the amount that faecal γ -globulin was able to bind; however, the lack of detectable radioactivity in tissues precluded the use of a lower dose.

Although coproantibodies are relatively resistant to proteolytic digestion¹², some antibody degradation may have

occurred during the faecal collection periods; thus the observed extent of ^3H -BaP binding to γ -globulin in excreted faeces may underestimate the binding that existed within the intestine. It is presumed, however, that the higher faecal antibody levels observed in immunised mice reflected higher intestinal antibody levels, although the possibility that they reflect reduced degradation is not formally excluded by the data. The binding observed in the faeces of control mice may represent the background level of the assay; alternatively, however, it is conceivable that these mice possess low level immunity induced by previous exposure to environmental PAHs which had combined with cellular macromolecules *in vivo* to form antigenic complexes.

Extrapolation of our data from mice and guinea pigs to humans suggests the feasibility of inducing antibody levels capable of binding, and perhaps neutralising carcinogens present in the environment in low, but not high, concentrations. Thus protection might be feasible against a strong carcinogen, such as BaP in the ng quantities generated by smoking a cigarette¹³, or the μg quantities ingested in a charcoal-broiled steak¹⁴. In contrast, little protection could be expected against a substance such as saccharin, currently under suspicion as a weak carcinogen, in persons exposed to the mg quantities contained in beverages. Similar stoichiometric considerations dictated the choice of the experiments described here as an initial means of assessing the potential efficacy of immunisation. The ability of immunisation to protect animals against carcinogenesis would constitute a more direct test, but such an experiment might be futile unless it entailed a very long testing period with large numbers of animals so as to permit the use of microgram doses of carcinogens. Thus, Creech and Franks, who immunised mice with dibenzanthracene, failed to observe significant protection against tumours induced by subsequent injection of 1 mg of this carcinogen^{15,16}. To bind this amount of dibenzanthracene completely, however, would have required each mouse to generate almost 300 mg of specific antibody molecules.

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Table 3 Effect of immunisation on BaP binding to faecal γ -globulin *in vitro* and *in vivo*

Immunising regimen	<i>In vitro</i> binding capacity for BaP of γ -globulin fractions of faeces (ng BaP per g faeces)*	% of administered dose recovered in γ -globulin fraction of faeces collected for 3 d after ^3H -BaP feeding†‡	
		per ml of faecal extract	per entire 3-day faecal collection
FMBAAA-BSA	0.64 $\pm$ 0.13	0.32 $\pm$ 0.05	3.1 $\pm$ 0.8
BSA	0.37 $\pm$ 0.08	0.16 $\pm$ 0.04	0.9 $\pm$ 0.1
None	0.32 $\pm$ 0.05	0.18 $\pm$ 0.03	1.7 $\pm$ 0.4

Values are expressed as mean $\pm$ standard error.

*Faeces were collected for 24 h from 11 FMBAAA-immunised, 9 BSA-immunised, and 10 unimmunised mice, starting 13 d after the last immunisation; they were homogenised in phosphate buffer, pH 6.5, 0.1 M (3 ml per g faeces), and particulate matter was removed by centrifugation. 100 μl of the clarified extract was then mixed with 1 ng of ^3H -BaP (Amersham-Searle, 26 Ci mmol⁻¹). After 40 min, 80 μl of rabbit anti-mouse γ -globulin (Cappel) was added, plus 8 μl of normal mouse serum. This latter material provided mouse γ -globulin in at least five fold excess of that present in the faecal extracts. This carrier γ -globulin ensured not only that a precipitate would form, but also that the quantity of immune precipitate would be similar in each sample, reducing the possibility that any non specific coprecipitation of ^3H -BaP that might occur would differ among the three treatment groups. The mixture was refrigerated for 18 h, and the precipitates were then washed by centrifugation in 20:1 PBS-dioxane and then twice in PBS, and were dissolved in NCS solubiliser for liquid scintillation spectrometry to determine how much ^3H -BaP had been precipitated with the γ -globulin. Faecal ^3H -BaP binding capacity was significantly greater in FMBAAA-immunised mice than in unimmunised mice ($P < 0.05$).

†The radioactivity measured in these experiments presumably includes BaP metabolites as well as unchanged BaP.

‡Eighteen days after the last immunisation, 6 FMBAAA-immunised, 5 BSA-immunised, and 6 unimmunised mice were fed ^3H -BaP by gavage (0.1 $\mu\text{g kg}^{-1}$ in 0.1 ml corn oil). Faeces were analysed as above for γ -globulin-bound radioactivity without the addition of exogenous ^3H -BaP. γ -Globulin bound ^3H -BaP excretion was significantly greater in FMBAAA-immunised mice than in either of the control groups ($P < 0.05$).

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Clones of alloreactive T cells

ONE of the basic tenets of the clonal selection theory is that antigen-specific precursor cells bear recognition structures directed towards a single antigenic specificity. The concept of clonal selection at the level of B-cell responses is widely accepted, but the proof of clonally distributed antigen receptors on T cells is lacking. Indirect evidence for clonally distributed T cell receptors has been produced in various ways (see ref. 1 for review), but functional clones of T cells have not been conclusively demonstrated. Using modifications of techniques previously described for isolation of clones of transformed cells² and for the production of colonies of T cells in soft agar³, we have succeeded in deriving clones of alloreactive murine T cells.

We selected long-term primed mixed leukocyte reaction (MLR)-positive cells as our source of potential clones. These primed responder cells (PRC) had been extensively studied^{4,5} and seemed to provide several possible advantages for our initial attempts: they were adapted to survival and propagation *in vitro* and were highly enriched for MLR-reactive cells⁴. In addition, we had some indication that there might exist clones of PRC-reactive to 'hybrid' histocompatibility antigens on (B6×A)_F₁ stimulator cells⁵.

We initially attempted to clone lymph node cells from strain A which had been primed *in vitro* to stimulator cells from strain B6, [A(B6)PRC] or (B6×A)_F₁ [A(B6A)PRC]. These PRC had been maintained in culture for several months before cloning. At 48 h after initiation of a mixed lymphocyte culture, we plated several different concentrations of PRC in the presence of stimulator cells. Colonies were picked after 1 week and, after suitable expansion (see Table 1), were assayed for alloreactivity, and aliquots of selected colonies were stained for cell-surface markers. All cells in the colonies assayed for surface markers were Thy 1-positive and surface Ig-negative (data not shown). Table 1 shows representative data from the first 100 colonies we assayed. (The alloreactivities of the parental cultures are included for comparison.) The predominant reactivity of all 50 of the colonies selected from plates seeded with A(B6A)PRCs was observed in response to re-stimulation with (B6×A)_F₁ stimulator cells. This supports our original premise that there are PRC with specificity for unique

F₁ MLR determinants⁷. Whereas most of the colonies showed quite restricted reactivities, a minority exhibited reactivities towards all three stimulator cell types, as does colony A(B6A)2-19. The finding in the 50 A(B6)PRC colonies (Table 1) that the majority of colonies exhibited responsiveness directed mainly towards B6 stimulator cells (without equal responses to (B6×A)_F₁ stimulator cells) was totally unexpected. Again, although the majority of these putative clones showed restricted reactivities, a minority exhibited significant reactivities toward all three stimulator cell types. There are at least two possible explanations of these data. Either we have isolated both clones and colonies containing cells from several alloreactive clones (that is, not truly cloned) or the different clones exhibit cross-reactivity for certain alloantigens (or both). As these PRC do not contain cytotoxic effector cells⁵, we were unable to access the question by cytotoxicity. Subcloning experiments are underway.

We followed the specificity of selected colonies as a function of time to see if the pattern of reactivity remained constant. Figure 1 presents the results of several assays carried out at 2-week intervals with cells from colonies A(B6A)1-1 and A(B6)1-13 (the parental culture reactivities are included at the initiation and termination of this time period for comparison). The reactivities of A(B6A)1-1 against (B6×A)_F₁ stimulator cells and the lack of significant response against B6 (or DBA/2) stimulator cells was maintained throughout this culture period. The increased reactivity of A(B6)1-13 against B6 cells compared with (B6×A)_F₁ (and DBA/2) cells was also maintained. To determine whether we were dealing with monoclonal responses in these two colonies, we assayed their responsiveness to the T-cell mitogens concanavalin A and phytohaemagglutinin (PHA) (to which both parental PRC populations responded) as well as to other hybrid stimulator cells. The results presented in Table 2 support the concept that we have obtained clones of alloreactive T cells: A(B6A)1-1 reacts only with (B6×A)_F₁ and (CBA×B6)_F₁ stimulator cells and not with B6, DBA/2 (DBA×B6)_F₁ or either T cell mitogen. A(B6)1-13 responds to B6 stimulator cells, much less to (B6×A)_F₁, (CBA×B6)_F₁ and (DBA×B6)_F₁, and not at all to DBA/2 or either mitogen.

These results support our previous suggestion that some clones of A(B6A)PRC can recognise unique F₁ MLR determinants⁷. Furthermore, they raise the possibility that there

Table 1 Proliferative responses of selected colonies of A(B6) and A(B6A) PRC

Responder cells	Stimulator cells			
	A/J	B6	B6×A	DBA/2
A(B6A) uncloned	2,458±1,032	17,771±1,032	32,210±480	8,431±63
A(B6A) 1-1	703±490	2,255±979	33,758±3,745	859±21
A(B6A) 1-8	1,537±1,420	1,304±249	42,642±4,575	2,492±344
A(B6A) 2-1	977±159	6,580±42	21,166±3,186	1,364±231
A(B6A) 2-19	1,677±930	3,430±661	43,099±1,026	15,992±2,051
A(B6) uncloned	2,301±506	19,592±4	21,908±246	9,724±1,163
A(B6) 1-2	1,299±372	34,992±1,433	36,997±4,591	36,637±2,929
A(B6) 1-13	787±400	13,027±1,174	2,185±675	1,356±124
A(B6) 2-10	788±308	17,771±113	9,204±478	3,261±565
A(B6) 2-13	1,238±178	33,536±1,973	10,672±408	3,612±606

Strain A lymph node cells were used as responder cells in mixed lymphocyte cultures (MLCs) and primed and re-stimulated with irradiated (3,300R) spleen cells from either strain C57/BL6(B6) or (B6×A)_F₁ hybrid mice as described previously⁴. The surviving responder cells (PRC) were serially re-stimulated in one generation⁵. Two days after the fifth serial re-stimulation 2×10^6 PRCs of both types and 60×10^6 irradiated (3,300R) stimulator cells were re-suspended in MLR medium (RPMI 1640 containing glutamine, antibiotics, 2-mercaptoethanol and 10% foetal calf serum (FCS))⁴ and seeded in soft agar (0.25%) over a base layer of 0.5% agar⁶. Colonies were picked 7 d later and placed in flat-bottomed microtitre wells (Falcon 3040) containing 0.2 ml fresh MLR medium and 1×10^6 irradiated stimulated cells. Six to seven days later, the colonies were transferred to Costar trays (Cluster 24, 3524 Costar) containing 2 ml fresh MLR medium and 10×10^6 stimulator cells. Five to six days later the colonies were transferred into tissue culture flasks (Falcon 3013) containing an additional 3 ml fresh MLR medium. Two days later aliquots of the colonies were assayed for reactivity in a standard I^H MLR (ref. 4) or expanded by restimulation in tissue culture flasks.

Table 1 shows representative results (as ³H thymidine incorporation) of MLR cultures in which 10×10^3 PRC were re-stimulated with 1×10^6 irradiated stimulator cells and collected at day 3 (ref. 4). The parental cultures (A(B6) and A(B6A)) were re-stimulated in an identical fashion and the results included for comparison.

Table 2 Proliferative responses of 'cloned PRC'

Stimulator cells	Responder cells			
	A(B6A) (c.p.m. + s.d.)	A(B6A) 1-1 (c.p.m. + s.d.)	A(B6) (c.p.m. + s.d.)	A(B6) 1-13 (c.p.m. + s.d.)
A/J	2,632 ± 137	969 ± 432	1,033 ± 63	863 ± 168
B6	19,921 ± 732	612 ± 195	64,558 ± 379	17,398 ± 2,399
(B6 × A)F ₁	39,608 ± 2,317	23,036 ± 5,452	63,015 ± 2,012	6,928 ± 237
DBA/2	11,321 ± 407	1,601 ± 157	22,454 ± 1,230	796 ± 203
CON A	22,034 ± 107	359 ± 165	11,734 ± 201	238 ± 33
PHA	8,423 ± 107	901 ± 106	8,431 ± 624	118 ± 3
(CBA × B6)F ₁	40,017 ± 316	29,997 ± 660	54,901 ± 7,497	9,300 ± 291
(DBA/2 × B6)F ₁	24,732 ± 97	636 ± 172	55,499 ± 1,537	3,014 ± 458

The results of re-stimulation of both parental cultures and two selected colonies (A(B6A) 1-1 and A(B6) 1-13) expressed as c.p.m. of incorporated ³H-thymidine (at day 3) in response to stimulator cells or T cell mitogens. 10⁴ PRC were re-stimulated with either 10⁶ irradiated (3,300R) stimulator cells or appropriate concentrations of both T cell mitogens (2 µg ml⁻¹ concanavalin A and 1/20 dilution of PHA.M)⁹.

exist unique homozygous MLR determinants and clones of cells (that is, A(B6)1-13) which recognise them. Finally, these results suggest that certain alloreactive T cells are not responsive to the T-cell mitogens concanavalin A or PHA.

The striking finding that all the colonies we assayed exhibited specificity for the priming stimulator cell was unexpected. Because of the high numbers of PRC seeded per plate, we expected various degrees of third-party reactivity in the colonies. From previous studies demonstrating apparent cross reactivities⁴, we would expect that the distribution of primary specificities among the cloned cultures would be the same as the specificity distribution within the bulk culture. The finding of single primary specificities (A(B6A)PRC colonies recognised (B6 × A)F₁ and

A(B6)PRC recognised B6 stimulator cells) in almost all of the 100 colonies assayed and the low cloning efficiency of seeded PRC raises one interesting possibility. It is conceivable that bulk MLR cultures allow triggering of many clones of T cells with low affinity (cross-reactive) receptors, as well as mitogen-responsive cells, due to the release of lymphokines from the immunocompetent interactions of the small number of cells with high affinity receptors. The interaction of cells with low affinity receptors and/or lymphokines may allow clonal expansion of such mitogen-responsive and low affinity receptor alloreactive T cells in bulk cultures. In soft agar, we trigger only alloreactive clones bearing high affinity receptors and inhibit the action of the lymphokines that would allow triggering of mitogen-responsive T cells and clones of T cells bearing cross-reactive, low affinity receptors. Thus, the colonies we pick from soft agar have primary responsiveness towards the priming stimulator cell; and the low responsiveness towards 'inappropriate' alloreactive cells we have observed is probably a function of the contaminant PRC that were in the area of the colony and were picked in the initial transfer and then expanded through the mechanism outlined above for cultures in suspension.

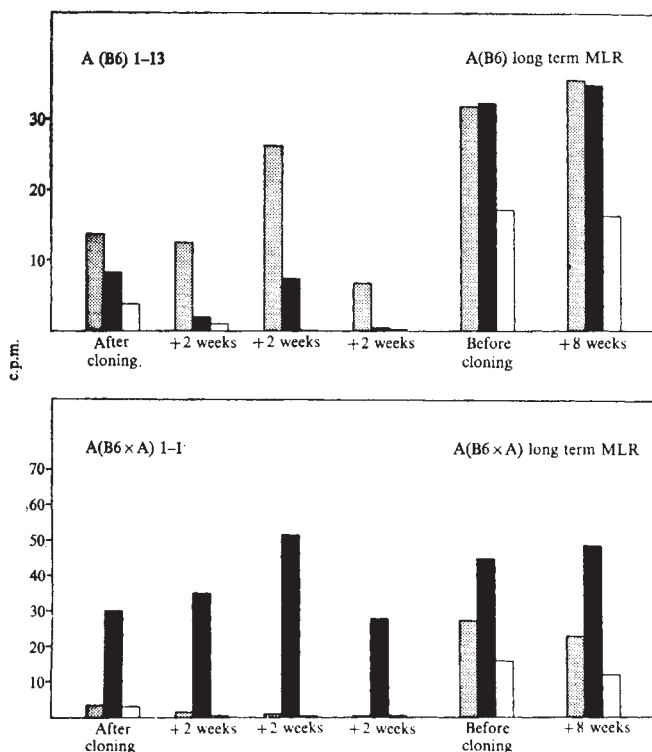
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Loss of Fc receptors for IgG from human T lymphocytes exposed to IgG immune complexes

SURFACE receptors for immunoglobulins (Ig) have been detected on mononuclear lymphoid cells of several animal species. They are present on monocytes, macrophages, T and B lymphocytes and on a third as yet poorly defined population of non-T non-B cells ('null' cells)¹. Early studies demonstrated mainly receptors for IgG, but binding of IgM and IgE to lymphoid populations has also been observed²⁻⁶. In humans the presence of surface receptors for either IgM or IgG molecules distinguishes two distinct T cell populations (T_M and T_G respectively) which have clearly defined functional

characteristics. T_M cells help B cell differentiation induced by pokeweed mitogen^{7,8}, whereas T_G cells suppress differentiation. T_G lymphocytes only suppress following interaction with IgG immune complexes, perhaps indicating an important role for IgG-Fc receptors in the regulation of humoral responses⁸. Another recognised role for IgG-Fc receptors is to mediate the antibody-dependent cell-mediated cytotoxicity (ADCC)^{9,10}. This latter activity is associated with both T and 'null' lymphocytes^{11,12}. We report here that interaction between IgG immune complexes and IgG-Fc receptors present on human T_G cells is followed by the irreversible disappearance of the Fc receptors. As a consequence of this 'modulation' T_G lymphocytes lose most of their cytotoxic activity in an antibody-mediated cell killing system.

Purified T cells forming rosettes with neuraminidase-treated sheep erythrocytes were isolated from human peripheral blood on Ficoll-Hypaque gradients. T_G and T_M were detected by rosette formation with ox erythrocytes (OE) coated with either IgG or IgM rabbit antibodies (IgG-OE and IgM-OE), and in some experiments cells rosetting with the one of the immune complex were further purified on density gradients^{2,7,8}; OE were subsequently removed by lysis with distilled water. Fc receptors present on such purified T_M and T_G cells were therefore exposed during cell isolation to either IgM or IgG immune complexes. The effect of this interaction on the capacity of cells to express their Fc receptors was then examined by rosetting with IgG-OE and IgM-OE. IgM receptors were detectable on the large majority of isolated T_M cells after overnight culture (82%, 91% and 86% in three different experiments), whereas very few purified T_G were able to bind IgG-OE even after 3 d in culture (Table 1). The failure to synthesise the IgG-Fc receptor was not a simple consequence of cell death. Thus 20 h after the original rosetting more than 95% of cells were viable (as judged by Trypan blue exclusion) and responsive to T-cell mitogens, as previously reported⁷. Furthermore, no significant alterations of other T-cell markers were observed. For example, most isolated T_G cells formed rosettes with sheep erythrocytes and were stained by a specific anti-T antiserum⁸.

We also investigated the possible blocking of Fc receptors by immune complexes persisting after the isolation procedure, such as fragments of erythrocytes coated with rabbit IgG antibody. Isolated T_G lymphocytes were examined before and after overnight incubation *in vitro*. Persistent rabbit IgG was revealed with a fluoresceinated goat anti-rabbit IgG⁸. Cells were also treated with trypsin in conditions allowing release of immune complexes without a loss of the trypsin-resistant Fc receptor for IgG¹³, and then tested by rosetting with IgG-OE. Freshly isolated T_G , after lysis of IgG-OE, did have detectable bound immune complexes which caused some inhibition of rosette formation with IgG-OE but trypsin treatment completely restored the capacity of T_G to rosette with IgG-OE (Table 1). After overnight incubation *in vitro*, however, the isolated cells were not positively stained with the fluorescent goat anti-rabbit IgG, and did not form rosettes with IgG-OE after treatment with trypsin (Table 1).

A similar effect on the expression of Fc receptors for IgG was induced by aggregated human IgG. In two experiments purified T-cell populations containing respectively 12 and 14% of cells forming rosettes with IgG-OE were cultured overnight with heat-aggregated human IgG (1.5 mg ml⁻¹) prepared according to Dickler¹³; after this treatment only 1 and 3%, respectively, of T-cells formed rosettes with IgG-OE (data not shown). Furthermore this negative result was not reversed by treatment of the lymphocytes with trypsin in conditions which specifically remove immune complexes.

Thus, in two different systems, interaction of IgG-Fc receptors with their ligand caused their irreversible loss from the lymphocyte surface. In contrast we could demonstrate resynthesis of IgG-Fc receptors after enzyme removal. Unfractionated T-cells in TC 199 medium were treated with pronase (1 mg ml⁻¹) for 30 min at 37 °C. At this stage the

Table 1 Modulation of IgG-Fc receptors induced by IgG immune complexes in purified human T_G populations

T_G population	% of cells forming-rosettes with IgG-OE	
	untreated	trypsin-treated
Freshly isolated	77,81,89	98,96,99
Cultured for 20 h	8,11,6	6,12,6

T-cells with Fc receptors for IgG (T_G) forming rosettes with ox erythrocytes coated with rabbit IgG antibodies (IgG-OE) were isolated from a purified T fraction by pelleting through two consecutive Ficoll gradients as previously described^{7,8}. The suspensions of isolated cell contained less than 1% non-rosetting cells. IgG-OE were removed by treatment with distilled water and lymphocytes were resuspended in medium 199 supplemented with 20% foetal calf serum^{7,8}. Cells were tested for rosetting with IgG-OE immediately or after incubation overnight at 37 °C, and with or without trypsin treatment (2 mg ml⁻¹ for 30 min). The values represent the results of three independent experiments.

lymphocytes failed to react with IgG-OE. A complete re-synthesis of IgG-Fc receptors was, however, noted within 8–12 h of culture *in vitro*¹⁴. Similar results were obtained in control experiments in which T lymphocytes, before treatment with pronase, were pelleted with OE and subjected to hypotonic shock. Thus, exposure to distilled water does not effect the capacity of T lymphocytes to resynthesise their Fc receptors. On the other hand, T_G cells which had been reacted with IgG immune complexes and then treated with pronase were not able to re-express their Fc receptors (data not shown). This suggests that contact with IgG immune complexes could act as a 'switch off' signal for synthesis and/or membrane insertion of the IgG receptors by T_G .

An immediate consequence of the phenomenon, easily demonstrable *in vitro*, was the sharp decrease of ADCC elicited by T-cell populations which had been previously reacted with IgG-OE complexes (Table 2). T-cell populations depleted of T_G by removing lymphocytes rosetting with IgG-OE no longer killed sensitised target cells, confirming that ADCC depends on the T_G subpopulation¹¹ (Table 2). However, these cells, when exposed to IgG complexes and cultured no longer killed target cells, presumably because of the loss of their IgG-Fc receptors. Finally, it is important to ask if this ligand-mediated irreversible modulation of IgG-Fc receptors has

Table 2 T-populations pre-exposed to IgG immune complexes lose most of their activity in an antibody dependent cytotoxic assay (ADCC)

T-cell population	K cell activity in ADCC assay	
	(% ⁵¹ Cr release)	
Unfractionated T	21,25	
T-depleted of T_G	3,4	
Unfractionated T exposed to IgG immune complexes	5,3	

Human T lymphocytes forming rosettes with neuraminidase-treated sheep erythrocytes were purified as previously described^{7,8}. One aliquot was depleted of T_G by removing cells rosetting with IgG-OE^{7,8}. Unfractionated T lymphocytes were either exposed to IgG immune complexes by centrifuging them with IgG-OE or pelleted with OE which were not coated with IgG, as control. Erythrocytes were lysed with distilled water and the various T cell suspensions, cultured for 20 h, were analysed for their capacity to lyse ⁵¹Cr-labelled target erythrocytes in a 18 h assay in the presence of anti-target antibody in accordance with Perlmann *et al.*¹⁵. The test was set up in microcultures with a final volume of 0.3 ml per tube. Each test tube contained 0.1 ml of lymphocytes (4×10^6 ml⁻¹), 0.1 ml of ⁵¹Cr-labelled OE (1×10^6 ml⁻¹) and 0.1 ml diluted rabbit IgG anti OE antibody. Controls in which lymphocytes were substituted by 5×10^6 unlabelled OE were included. The incubation medium used was TC 199 supplemented with 7.5% foetal calf serum. Triplicate tubes were incubated at 37 °C in 5% CO₂, 95% air and release of isotope into the culture medium was given by counting both supernatants and pellets in a scintillation counter. The percentage of ⁵¹Cr release is used as a measure of the lysis. All the values reported are adjusted by subtracting the percentage of ⁵¹Cr release in the control tubes (<2.5%).

significant consequences in the normal immune response. Interaction between immune complexes and the Fc receptor on the surface of suppressor T_G cells may greatly slow down the turnover of the receptor itself and render these cells not susceptible, or poorly susceptible, to further activation by immune complexes. This may represent a mechanism to prevent an excess of suppression. In addition, a 'switch off' mechanism of this type may play a part in the impaired immunological reaction observed in diseases involving circulating immune complexes. Reduced ADCC has been reported in patients affected by systemic lupus erythematosus¹⁶, and we found that most patients of this diagnostic group had very low proportions of T-cells forming rosettes with IgG-OE (unpublished observations). Both of these findings may indeed be related to the "switch off" of IgG receptors by the immune complexes present in great amounts in this immunological disorder. We thank Dr Michael Parkhouse for helpful discussions.

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Suppressor T-cell memory

MEMORY is a fundamental characteristic of immunological phenomena; it is manifested by enhanced responsiveness upon re-exposure to antigen, and its specificity forms the basis of the clonal selection concept. In collaborative antibody responses the development of memory has been demonstrated in both the T helper (T_H) cell and the B cell populations¹, whereas in cell mediated reactions such as delayed hypersensitivity, allograft rejection, cytotoxicity and graft-versus-host responses, memory is a property of the T cell subpopulation mediating each type of response. Specific suppression of antibody production by a subpopulation of T cells is now well documented²⁻⁴. The importance of suppressor T (T_S) cells in immune homeostasis is highlighted by their demonstration during normal immune responses⁵ as well as following tolerance induction⁶. After primary immunisation, however, T_S cells are detectable for only a limited period of time and do not prevent the emergence of long-lived T_H and B memory cells. The parallel development of memory T_S cells would therefore be highly advantageous if these cells are to be effective in the regulation of secondary immune responses. Furthermore, the potential value of T_S in the maintenance of active tolerance to self antigens would be enhanced if a pool of memory cells existed which could be rapidly recruited to abort incipient autoimmune responses. The experiments reported here demonstrate the presence of specific long-

lived memory T_S cells in mice exposed to the protein antigen, human γ -globulin (HGG).

Mice of the CBA/Ca/T6 strain were used throughout. Suppressor activity of lymphoid cells was assayed by testing their capacity to abrogate the adoptive anti-hapten antibody response to dinitrophenylated HGG (DNP.HGG) as described previously⁷. Briefly, spleen cells from mice primed separately to HGG and DNP keyhole limpet haemocyanin (DNP.KLH) were transferred with or without putative T_S cells into heavily irradiated syngeneic recipients. The latter were then challenged with DNP.HGG and the number of anti-DNP plaque-forming cells (PFC) in the spleen enumerated on day seven. In preliminary experiments, evidence was sought for generation of T_S cells during a primary immune response to HGG. Mice were immunised with 500 μ g alum-precipitated HGG (aHGG) and 2×10^6 heat-killed *Bordetella pertussis* organisms. When spleen cells from these donors were transferred 7-14 d later, together with DNP-primed and HGG-primed cells, 80% suppression of the anti-DNP PFC response was observed (Fig. 1). The cell responsible was shown to be a T cell and the effect was specific for the inducing carrier. These results indicated that T_S cells were generated in parallel with T_H cells following exposure to antigen in immunogenic form.

The development of T_S cell memory in mice given aHGG was tested by challenging them with deaggregated antigen (dHGG) four or more weeks after primary immunisation. dHGG was used because it had been previously demonstrated to exert a preferential effect on T_S without activating T_H cells⁷. It was therefore predicted that this manoeuvre would optimise the chance of eliciting an enhanced degree of suppression. As shown in Fig. 1, the suppressor activity of spleen cells from such mice was significantly greater than that of cells from animals given dHGG or aHGG alone. The effector cell responsible for the enhanced suppressive effect was shown to be a T cell with surface characteristics (Ia^+ , $Ly-2/3^+$, $Ly-1^-$) identical to those of T_S studied previously in this⁸ and other systems⁹.

The difference in magnitude of the 'primary' and 'secondary' T_S cell responses was consistent with the presence of memory T_S cells. To confirm this, it was necessary to establish that the secondary T_S response had an accelerated onset compared with the primary T_S response, and that it was more profound, of longer duration and mediated by fewer cells. The kinetics of primary and secondary T_S responses were compared by measuring suppression with transfer at different times after administration of HGG to naïve or preimmunised donors. Induction was dramatically accelerated in the secondary response (Fig. 2), marked suppression being evident by day two and reaching a peak by day three, whereas primary suppression was not detected until day five. Furthermore, the duration of the secondary response was shown to be greatly prolonged, suppression still being apparent in transfer 12 weeks after induction, in contrast to 4-6 weeks in the case of the primary response.

The third characteristic feature of immunological memory is that an equivalent response is elicited by fewer cells after re-exposure to antigen. To demonstrate this, varying numbers of spleen cells from naïve or preimmunised donors given dHGG were transferred into irradiated hosts together with standard numbers of DNP-primed and HGG-primed spleen cells. Figure 3 shows that 5-10 times fewer cells from sensitised donors were required to produce an equivalent degree of suppression.

The longevity of T_S memory for HGG was examined by injecting dHGG into groups of mice immunised with HGG at various times beforehand. When the suppressive effect of spleen cells from these animals was compared with that of control cells from age-matched naïve animals given dHGG at the same time, specific enhancement of suppression was shown to persist for at least nine months after primary immunisation. (Donors in the experiment shown in Fig. 1

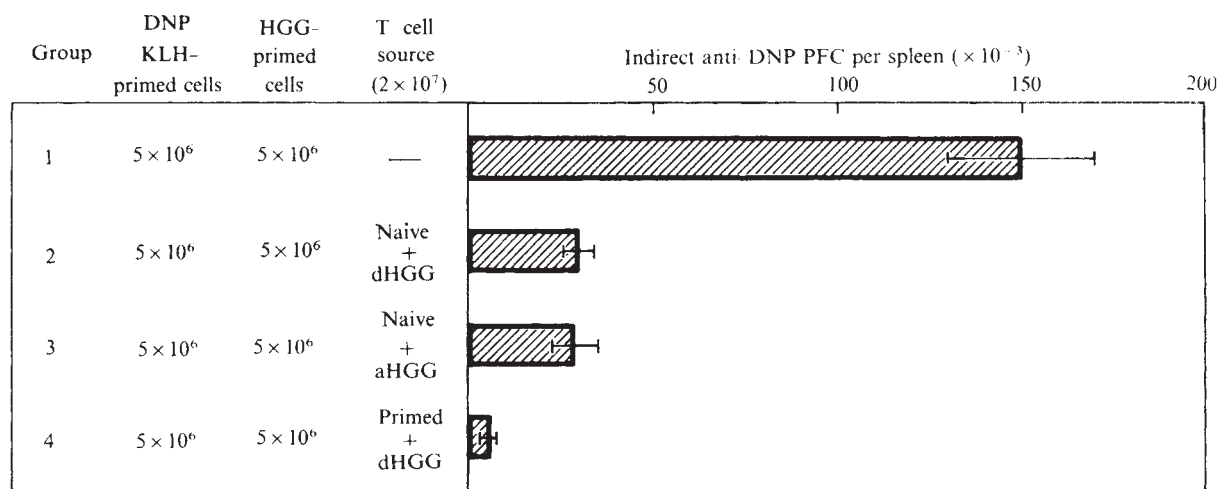
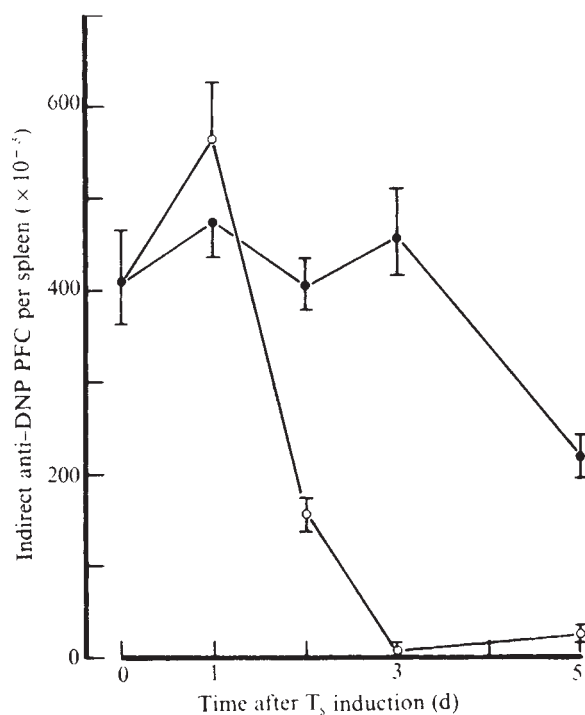


Fig. 1 Demonstration of primary and secondary suppressor responses in a collaborative anti-hapten assay system. Hapten-primed cells were obtained by intraperitoneal injection of CBA/Ca/T6 mice with 250 μ g dinitrophenylated keyhole limpet haemocyanin (DNP₈₀₀KLH) mixed with 2×10^9 killed *Bordetella pertussis* organisms. Carrier-primed cells were obtained by immunisation with human IgG (HGG) as described in the text. Spleen cells from such donors were used 3 or more weeks after priming. 'Primary' suppressor cells were induced by intraperitoneal injection of naive animals either with 3 mg HGG deaggregated by ultracentrifugation (group 2) or with 500 μ g alum-precipitated HGG (aHGG) and *B. Pertussis* (group 3). Induction of putative 'secondary' suppressor cells was by rechallenge of mice primed to aHGG at least 4 weeks previously with 3 mg dHGG. Spleen cells were collected 7–14 d later and assayed for suppression in an adoptive transfer system. For this purpose, 2.5×10^7 cells were injected intravenously into irradiated (700 rad from a ^{60}Co source) syngeneic recipients together with 5×10^6 HGG-primed spleen cells and 5×10^6 DNP.KLH-primed cells. Each recipient was then challenged with 500 μ g DNP₁₀HGG and the collaborative anti-DNP response measured 7 d later by splenic plaque forming cell (PFC) assay¹². The control response (primed cells alone, group 1) was 80% suppressed by the addition of cells from animals given dHGG or aHGG (groups 2 and 3). By contrast, suppression by spleen cells from primed donors rechallenged with dHGG was significantly increased to 97% (group 4).

were immunised nine months before administration of dHGG.) Thus, T_8 memory cells appear to be long-lived, and, by analogy with T_H memory cells, probably reside in the recirculating T_2 cell pool. Reactivation of suppression has been studied by Eardley and Sercarz¹⁰ who were able to show recall of specific suppression in an *in vitro* system,

Fig. 2 Kinetics of induction of primary compared with secondary suppressor responses. Normal ($\bullet$) mice or mice immunised with aHGG 12 weeks previously ($\circ$) were injected with 3 mg dHGG. Spleen cells collected 0–5 d later were transferred into 2 groups of 6 irradiated hosts at a dose of 2.5×10^7 cells per recipient, and assayed for suppression as described in Fig. 1 legend.



but the response lacked the secondary augmentation characteristic of memory, probably because of a simultaneous activation of memory T_H cells. This problem has been circumvented in our experiments by preferential recruitment of memory T_8 cells with antigen in the deaggregated form.

In view of the presence of high levels of anti-HGG antibody in preimmunised donors, and the known importance of immune complexes in regulation of immune responses, it

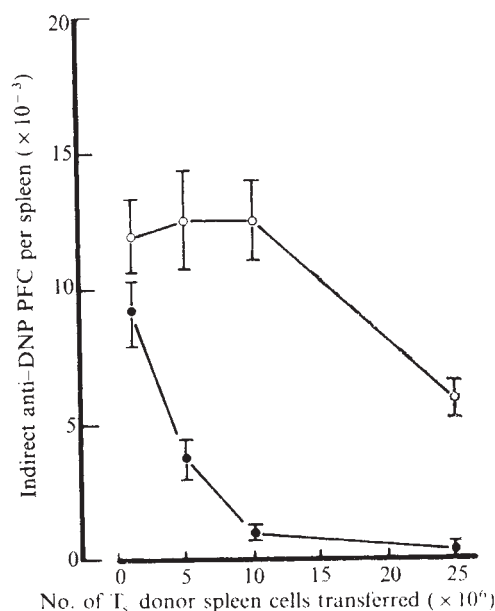


Fig. 3 Comparison of potency of cells from primary and secondary T_8 donor. Normal mice ($\circ$) or mice immunised with aHGG 8 weeks previously ($\bullet$) were injected intraperitoneally with 3 mg dHGG. Spleen cells were collected on day 7 and varying numbers transferred into groups of six irradiated recipients. Each recipient was then given 5×10^6 DNP-primed and 5×10^6 HGG-primed spleen cells, and challenged with 500 μ g DNP₁₀HGG. Splenic anti-DNP PFC responses were assayed 7 d later.

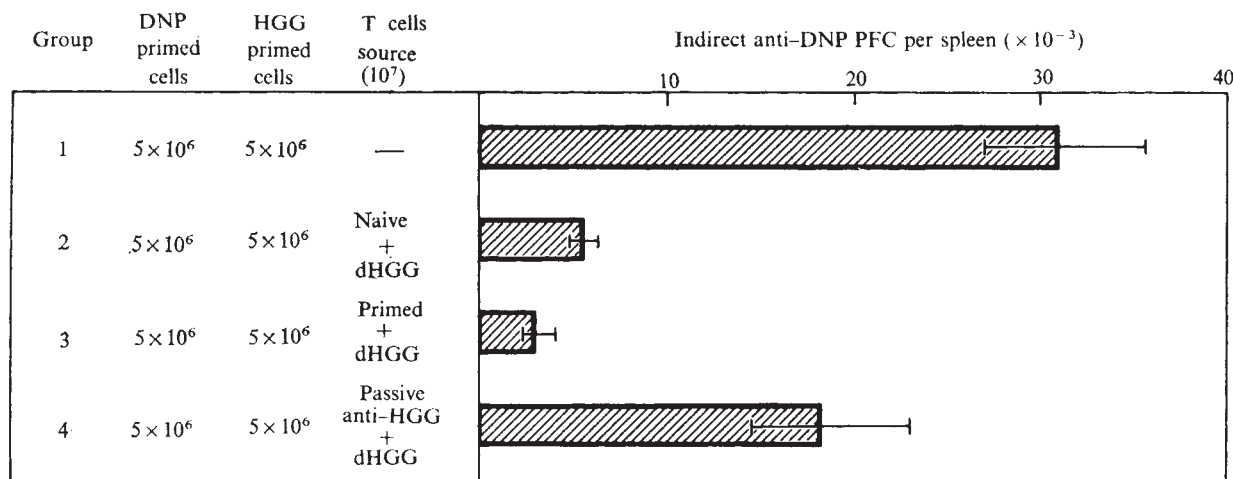


Fig. 4 Lack of evidence of a role for antigen-antibody complexes in the induction of the secondary suppressor response. T_s cells were induced by injecting normal mice (group 2) or mice immunised with HGG 8 weeks previously (group 3) with 3 mg dHGG intraperitoneally. Their suppressive capacity was compared with that of cells from mice given 200 µg purified anti-HGG antibody intravenously followed 24 h later by 3 mg dHGG intraperitoneally (group 4). The serum anti-HGG titre (measured by haemagglutination) immediately before dHGG injection was comparable to titres found in previously immunised animals. Spleen cells from the above three sources were harvested on day 11, transferred (10⁷ cells per animal) into groups of six irradiated recipients, and assayed for suppression as before.

was essential to exclude a role for them in induction of the augmented secondary suppressor response. Normal mice were therefore given sufficient purified anti-HGG antibody to achieve comparable serum levels to those found in immunised animals. Twenty-four hours later, they were injected with a standard dose of dHGG. After a further period of seven days spleen cells were transferred into irradiated recipients together with DNP- and HGG-primed cells, and challenged with DNP.HGG. Donor mice given dHGG but not anti-HGG antibody served as a source of control cells. As shown in Fig. 4, the suppressive activity of cells from mice pretreated with anti-HGG antibody was less pronounced than that of cells from animals given dHGG alone, indicating that circulating immune complexes are not responsible for generation of the secondary T_s response observed in previously immunised mice.

Taken together, the findings described above strongly suggest the existence of memory T_s cells. This is consistent with a fundamental role for these cells in the regulation of immune responses, and may be an important factor in the maintenance of a stable immunological network¹¹. Furthermore, a memory T_s pool may provide an efficient surveillance mechanism whereby any potential immune response to self antigens could be rapidly aborted.

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Collagen required for proliferation of cultured connective tissue cells but not their transformed counterparts

THE proline analogue, *cis*-hydroxyproline, when incorporated into collagen, prevents collagen secretion¹ and also inhibits the growth of fibroblasts *in vitro*². We describe here studies on the mechanism of action of *cis*-hydroxyproline on cell proliferation of primary cultures of murine connective tissue cells and of their spontaneously transformed, tumorigenic counterparts. We find that the analogue inhibits the attachment, spreading and proliferation of the connective tissue cells and that these effects are completely reversed when the cells are grown on collagen-coated dishes. The tumorigenic cells are less affected by *cis*-hydroxyproline even though their collagen synthesis is inhibited to a similar extent to the connective tissue cells. These results suggest that the presence of a collagen matrix is required for the growth of some connective tissue cells *in vitro* but not for the growth of their tumorigenic counterparts.

Murine connective tissue cells were prepared by injecting 3 ml of a 0.5% trypsin, 0.5% collagenase solution subcutaneously into 6-week-old BALB/c mice. The injection produced a subcutaneous bulla which after 45 min was injected with the Dulbecco modification of Eagle's minimal essential medium containing 10% foetal calf serum, and aspirated. The cells obtained from three mice were cultured in T75 flasks and reached confluency within seven days. After 15 passages *in vitro*³, transformed cells appeared which were able to produce tumours in syngeneic mice after subcutaneous injection (10 tumours per 10 syngeneic mice using this cell strain). Histological analysis of the tumours indicated that they were fibrosarcomas.

Cis-hydroxyproline was added to newly seeded cells or to cells in the log phase of proliferation as described by Kao and Prockop². Concentrations of *cis*-hydroxyproline from 15 to 400 µg ml⁻¹ were tested, and 40 µg ml⁻¹ was the minimum concentration required to inhibit cell proliferation. At this concentration, the effect was noticeable 24 h after addition of the analogue (Fig. 1a). The mechanism of the inhibition was studied by experiments in which the cells were grown on culture plates coated with 1 µg cm⁻² of native type I collagen. Prior coating of the plates with collagen reversed the inhibitory effect of the *cis*-hydroxyproline on cell growth

(Fig. 1a), while untreated cells grown on the collagen-coated plates showed a growth pattern similar to that of cells grown on a plastic surface. Fibrin-coated plates did not reverse the effect of *cis*-hydroxyproline (data not shown). The growth of the transformed, tumorigenic connective

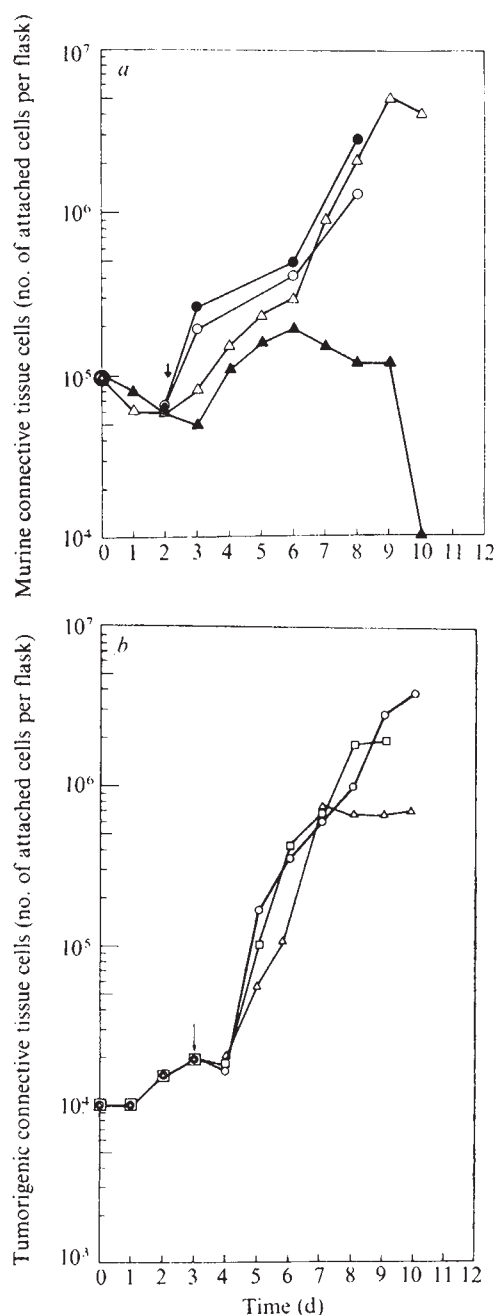


Fig. 1 The effects of *cis*-hydroxyproline on the proliferation of murine connective tissue cells and their spontaneously transformed counterparts on plastic and collagen-coated dishes. *a*, Connective tissue cells were prepared by enzymatic digestion of subcutaneous tissue from 6-week-old mice. Passage-three cells were plated at low density (6×10^3 cells per 35 mm culture dish) in Dulbecco's medium supplemented with 10% foetal calf serum, 100 units ml^{-1} of penicillin, 100 $\mu\text{g ml}^{-1}$ of streptomycin and 0.1 mg ml^{-1} ascorbic acid. Type I collagen was prepared from lathyritic rat skins¹ and coated on bacteriological plates as previously described. *Cis*-hydroxyproline (40 $\mu\text{g ml}^{-1}$) was added to the designated cultures at the time indicated by the arrow. Some cultures were grown with *cis*-hydroxyproline on either collagen-coated (△) or on untreated plastic dishes (▲). Control cultures were grown on either collagen-coated plastic (○) or on untreated plastic (●). *b*, The transformed cells were inoculated at low density and 0, 30 or 100 $\mu\text{g ml}^{-1}$ of *cis*-hydroxyproline was added at the time indicated by the arrow. The control (●), 30 $\mu\text{g ml}^{-1}$ (□) and 100 $\mu\text{g ml}^{-1}$ (○) treated cultures were grown only on plastic.

Table 1 Effect of *cis*-hydroxyproline pretreatment on attachment of connective tissue cells to plastic

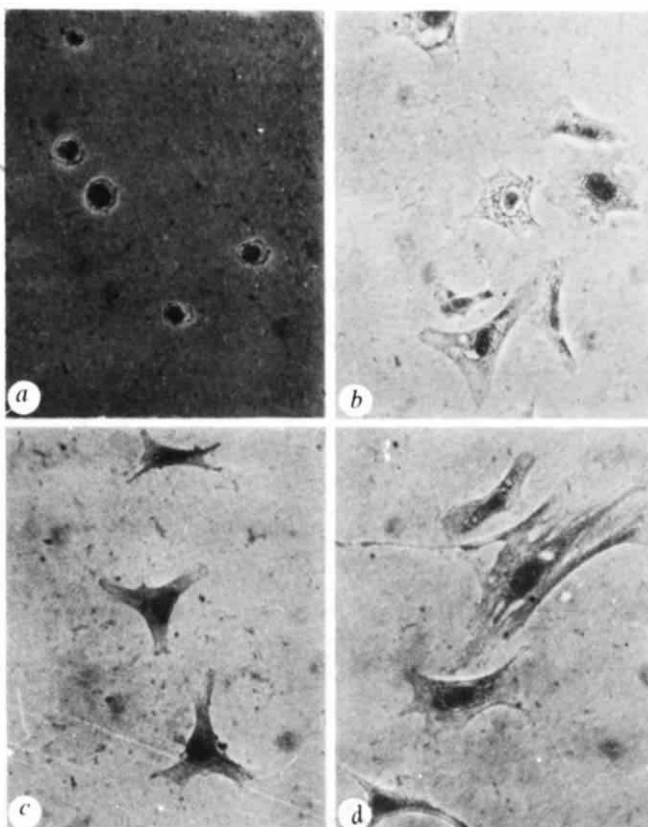
		% Reduction in no. of attached cells between <i>cis</i> -hydroxyproline treated and control cells ($\pm$ s.d.)
Non-tumorigenic connective tissue cells	Collagen	3.0 ± 2.0
	Plastic	34.0 ± 3.3
Tumorigenic connective tissue cells	Collagen	5.0 ± 3.0
	Plastic	4.0 ± 1.0

Cell monolayers grown in T75 flasks were trypsinised and inoculated onto T75 flasks in media with or without *cis*-hydroxyproline (100 $\mu\text{g ml}^{-1}$). After 20 h these pretreated cells were trypsinised and equal numbers inoculated on plastic or collagen-coated plates in 2% foetal calf serum with 100 $\mu\text{g ml}^{-1}$ of *cis*-hydroxyproline for study of the rate of attachment, as previously described^{5,8}. By 60 min the plateau region of the attachment curve was reached. Values expressed above reflect % reduction in proportion of attached cells (mean of three experiments) at 120 min.

tissue cells was markedly less inhibited by *cis*-hydroxyproline (Fig. 1b), and the cells remained attached and continued to proliferate after prolonged exposure (20 d) to the analogue even at a dose of 100 $\mu\text{g ml}^{-1}$.

The effect of *cis*-hydroxyproline on cell attachment and spreading was studied. Non-tumorigenic cells were pre-incubated with *cis*-hydroxyproline for 20 h then removed by trypsin, and their rate of attachment on plastic and collagen-coated dishes was measured. This treatment sig-

Fig. 2 Effect of *cis*-hydroxyproline pretreatment on morphology of non-tumorigenic connective tissue cells 6 h after inoculation on collagen or plastic. Cells pretreated with 100 $\mu\text{g ml}^{-1}$ of *cis*-hydroxyproline for 40 h, and identical numbers of untreated cells were inoculated onto collagen-coated dishes or untreated dishes (in Dulbecco's MEM plus 10% foetal calf serum, 0.1 mg ml^{-1} ascorbic acid and 40 $\mu\text{g ml}^{-1}$ *cis*-hydroxyproline). The pretreated cells which attached on plastic (*a*) were slow to spread but on collagen (*b*) they attached, spread and proliferated. Control cells attached and spread on both plastic (*c*) and collagen (*d*). (Magnification $\times 200$).



nificantly reduced the proportion of non-tumorigenic cells attaching to plastic in 2% serum after 120 min but had no effect on their ability to attach to collagen (Table 1). In contrast, the attachment rate and morphology of the tumorigenic cells on plastic or collagen was not altered by the same exposure to *cis*-hydroxyproline. Non-tumorigenic cells pretreated with the analogue spread more slowly on plastic than did untreated control cells (Fig. 2). The cells which failed to spread did not proliferate unless they were plated on collagen-coated dishes, where they both spread and proliferated (Fig. 2).

Collagen synthesis was measured in the two cell types before and after analogue exposure. Subconfluent cultures were labelled with ^{14}C -glycine, and collagen synthesis was estimated by measuring the proportion of labelled protein solubilised by purified bacterial collagenase (modified method of Diegelman and Peterkofsky⁶). The amounts of collagenase-sensitive proteins synthesised by both cell types were inhibited to a similar extent after exposure to *cis*-hydroxyproline (Table 2). Thus, it seems that the tumorigenic cells responded to the proline analogue with a similar reduction in collagen production to that of the non-tumorigenic cells. From separate experiments⁷, both cell types have been found to synthesise type I and III collagen, but the tumorigenic cells make one-fifth as much total collagen as the connective tissue cells.

Studies by Klebe⁸ have shown that many fibroblast cells attach to collagenous substrates by surface-associated glycoproteins (LETS). Cell attachment to a substrate *in vivo* has been defined into the phases of contact, initial attachment, and progressive attachments leading to spreading⁹. Our data suggest that the *cis*-hydroxyproline acts to inhibit murine connective tissue proliferation by preventing collagen deposition on the plastic substrate and thus preventing one or more of the phases of cell attachment, spreading and growth. Transformed connective tissue cells are resistant to inhibition by the proline analogue because they may not be as dependent on a collagen matrix for adherence and growth. From other studies, the tumorigenic cells are known to make approximately 80% less collagen (as a proportion of their total protein synthesis) and show reduced

secretion of LETS protein⁷. This supports the suggestion that these cells may be a subpopulation of the original primary explant which adhere, spread and grow at least partially independently of their collagen matrix, a property possibly related to their tumorigenicity.

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Spontaneous murine B-cell leukaemia

THE discovery of thymus-derived (T) and bone marrow-derived (B) specific cell-surface markers in normal lymphocyte populations led to extensive studies of the cell lineage and differentiation pathways of various human and murine lymphoid neoplasms. Most spontaneous lymphomas and lymphocytic leukaemias in mice are members of the T-lymphocyte lineage¹. Although B-lymphocyte tumours have been reported in mice, these neoplasms were induced experimentally, using carcinogens or Abelson virus^{2–5}. We report here the characteristics of a spontaneous B-cell leukaemia derived from, and passaged in BALB/mice. This tumour seems to be analogous to human chronic lymphocyte leukaemia (CLL), and may provide a useful model for the study of this disease.

During a study of the effects of total lymphoid irradiation (TLI) on the number and function of T and B cells among peripheral blood lymphocytes (PBL) in BALB/c mice, we examined blood samples of a control group of six normal, unirradiated mice at monthly intervals for over a year. All of these mice were bred in pathogen-free conditions (Department of Radiology, Stanford University), but were kept in conventional animal rooms during our experiments. One female BALB/c mouse in the control group showed a marked B lymphocytosis at 24 months of age, and an unexpectedly low proliferative response to T-cell mitogens. Table 1 shows that the absolute lymphocyte count of this mouse was $200,000\text{ mm}^{-3}$. All cells had the morphological appearance of small to medium lymphocytes on a Giemsa-stained blood smear (Fig. 1). The cells bore surface immunoglobulin (Ig), but no Thy 1.2 antigen. This haematological picture of marked B lymphocytosis is analogous to that observed in CLL (ref. 6). *In vitro* stimulation of PBL with two T-cell mitogens, phytohaemagglutinin (PHA) and concanavalin A (con A), produced no incorporation of ^3H -thymidine above background levels (Table 1). On the other hand, stimulation with a B-cell mitogen, lipopolysaccharide (LPS), produced a vigorous response which was considerably greater than that observed with normal BALB/c controls (Table 1).

At the time of killing, the spleen weighed 4.6 gm (normal spleen weight $\sim 0.1\text{ gm}$) and yielded approximately 5×10^9

Table 2 Effect of *cis*-hydroxyproline on synthesis of non-dialysable collagenase-sensitive ^{14}C -glycine labelled protein

		% Of collagenase-sensitive ^{14}C -glycine labelled proteins ($\pm$ s.d.)	
		Control	<i>cis</i> -Hyp
Non-tumorigenic connective tissue cells	Cell layer	10.0 ± 1.0	4.0 ± 0.8
	Media	55.0 ± 5.0	34.0 ± 3.0
Tumorigenic connective tissue cells	Cell layer	11.0 ± 1.5	4.0 ± 1.0
	Media	25.0 ± 2.0	9.0 ± 4.0

Medium was removed from T25 flasks containing 80% confluent cells. Two ml of serum-free Eagle's minimal essential medium containing $40\text{ }\mu\text{g ml}^{-1}$ *cis*-hydroxyproline (for the test culture), 0.1 mg ml^{-1} ascorbic acid, 0.1 ml gentamicin, $50\text{ }\mu\text{Ci}$ ^3H -leucine (50 Ci mmol^{-1}) and $50\text{ }\mu\text{Ci}$ ^{14}C -glycine (106 mCi mmol^{-1}) were added and incubated for 4 h at 37°C . The medium was then removed and the cell layer washed with phosphate buffered saline. The medium plus the wash were combined and dialysed. The cell layer (containing extracellular and intracellular proteins) was removed with a rubber policeman, suspended in PBS, sonicated and dialysed. Total ^{14}C -glycine and ^3H -leucine incorporation into protein by the tumorigenic cells was approximately 30% and 80%, respectively, of the amount synthesised by the connective tissue cells. Samples were aliquoted such that in each case 10^6 c.p.m. of non-dialysable ^{14}C -labelled protein was lyophilised and digested with purified bacteriological collagenase for 3 h at 37°C (form III, Advance Biofactures) followed by a TCA (10%), tannic acid (5%) precipitation. Differences in soluble and precipitable ^{14}C -radioactivity before and after collagenase treatment were used to compute % of collagenase-sensitive labelled proteins. Values expressed are the mean of four replicates from the same experiment for cells grown under control conditions or treated with *cis*-hydroxyproline ($40\text{ }\mu\text{g ml}^{-1}$) for 24 h.

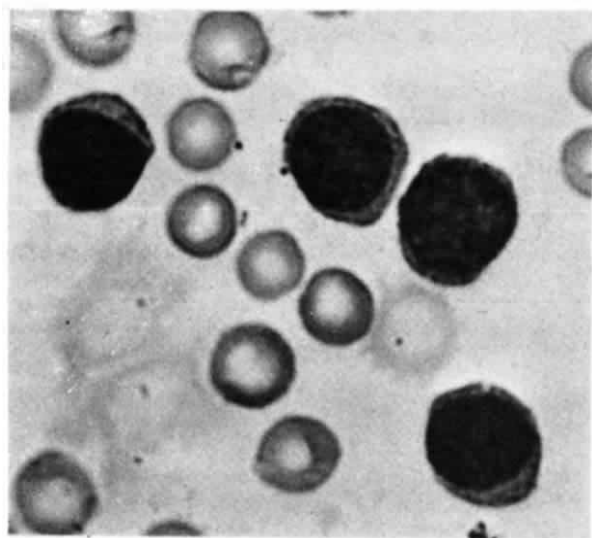


Fig. 1 Peripheral blood smear obtained from a BALB/c mouse which developed a spontaneous B-cell leukaemia at 24 months of age. Giemsa stain $\times 1,055$.

nucleated cells (normal spleen cells $\sim 10^8$ cells). Cells from the lymphoid tissues of this mouse were transferred intravenously, intraperitoneally, and subcutaneously to both unirradiated neonatal and adult mice, as well as to sublethally irradiated (500 rad) adult BALB/c mice. Recipients

given 5×10^6 PBL or spleen cells by each route developed a lymphocytosis, which was first detected approximately 3 months after intravenous cell transfer. Over 90% of all recipients showed a marked lymphocytosis ($> 20,000$ cells mm^{-3}) by 4 months. Table 2 shows a representative examination of the PBL of an irradiated recipient. The absolute lymphocyte count was 128,000 (small to medium cells) and 85% of these bore surface Ig.

In vitro stimulation of these PBL with PHA and con A again failed to induce incorporation of ^3H -thymidine. The response to LPS, however, was more than 20-fold greater than that of normal controls (Table 2). Autopsy of the irradiated recipients killed more than 3 months after cell transfer showed marked splenomegaly and lymphadenopathy. Spleens yielded as many as 9×10^8 nucleated cells. The histopathology showed replacement of the normal splenic and lymph node architecture with a homogeneous infiltrate of small to medium lymphocytes, and was consistent with a lymphocytic leukaemia or lymphoma. The marrow also showed a lymphocytic infiltrate which was not as extensive as that observed in the spleen. We have chosen to call this first reported example of a spontaneous murine B-lymphocyte leukaemia the BCL₁ tumour line.

The BCL₁ cells have now been successively passaged five times by way of intravenous injection of 5×10^6 spleen cells to unirradiated, and to sublethally irradiated adult BALB/c mice. Table 2 shows that the cell surface and *in vitro* functional characteristics of PBL from a representative recipient of the second passage were similar to those of the spontaneous tumour. The monoclonality of PBL from the

Table 1 Characteristics of peripheral blood lymphocytes in a single BALB/c mouse with spontaneous lymphocytosis

	BALB/c with lymphocytosis	Normal BALB/c
1. Absolute lymphocyte count mm^{-3}	200,000	5,200 $\pm$ 300
2. % Thy-1.2 lymphocytes	0	60 $\pm$ 8†
3. % Ig-positive lymphocytes	100	30 $\pm$ 9†
4. ^3H -Thymidine uptake of unstimulated lymphocytes	2,807 $\pm$ 222*	628 $\pm$ 114
^3H -Thymidine uptake of PHA ($1 \gamma \text{ ml}^{-1}$) stimulated lymphocytes	853 $\pm$ 122	46,347 $\pm$ 4,049
5. ^3H -Thymidine uptake of unstimulated lymphocytes	6,431 $\pm$ 528	1,302 $\pm$ 346
^3H -Thymidine uptake of con A ($2.5 \gamma \text{ ml}^{-1}$) stimulated lymphocytes	5,462 $\pm$ 708	49,366 $\pm$ 4,477
3. ^3H -Thymidine uptake of unstimulated lymphocytes	2,316 $\pm$ 162	2,497 $\pm$ 150
^3H -Thymidine uptake of LPS ($10 \gamma \text{ ml}^{-1}$) stimulated lymphocytes	136,872 $\pm$ 8,871	11,308 $\pm$ 44

1. Peripheral blood was diluted in acetic acid (2%) and nucleated cells were counted in a standard haemocytometer. Absolute PBL counts were calculated by multiplying the percentage of lymphocytes in blood smears treated with Wright's stain, and the nucleated (white) blood cell count. Mean normal values for PBL, and leukocytes (WBC) were obtained from pooled blood samples taken from a single group of 12 control mice on 14 different occasions during a 6-month period.

2. The percentage of T cells was determined using a dye-exclusion microcytotoxicity assay with a non-congenic anti-Thy 1.2 antiserum (AKR/J anti-C3H/He thymocyte antiserum)⁷. PBL purified on a Ficoll-Hypaque gradient⁸ were washed twice in PBS, and contaminating erythrocytes were lysed with ammonium chloride. The lymphocytes were resuspended in medium 199 containing 5% heat-inactivated foetal calf serum (FCS) to a concentration of $2 \times 10^6 \text{ ml}$. A sample (10 μl) of the lymphocyte suspension was incubated with 10 μl of anti-Thy/1.2 antiserum (1:10) for 30 min at room temperature. Guinea pig complement (5 μl) (final concentration 1:5) was added for an additional 45 min at room temperature. The cells were spun at 250g for 5 min and resuspended in 15 μl of medium 199. Trypan blue was added before each reading⁹. Viability of lymphocytes was determined in triplicates and the cytotoxic index was calculated using normal rabbit serum controls. The mean normal values were obtained from 20 pooled blood samples of 12 BALB/c mice over a period of 6 months.

3. The percentage of Ig-bearing cells was determined by a two-stage immunofluorescent-staining procedure using a polyvalent rabbit-anti-mouse Ig, and a fluorescein-conjugated goat-anti-rabbit Ig antiserum (Melyo Laboratories) as described previously¹⁰. PBL were stained in suspension, and smeared on to glass slides. The percentage of fluorescent cells was determined in three different experiments after counting a total of 100–200 small cells at 400 magnification using a Zeiss microscope with a tungsten light source and fluorescein isothiocyanate excitation filter. The mean normal values were obtained from 20 pooled blood samples of 12 BALB/c mice over a period of 6 months.

4. The proliferative response of PBL from BALB/c mice to T- and B-cell mitogens was measured by ^3H -thymidine uptake as described previously⁹. Response to phytohaemagglutinin (PHA): 1×10^5 purified PBL were aliquoted into 0.2 ml flat bottom microculture wells (Falcon 3040) containing RPMI 1640 supplemented with FCS (10%), glutamine (2mM), penicillin (100 U ml^{-1}), and streptomycin (100 $\mu\text{g ml}^{-1}$) in triplicate. PHA (purified-phytohaemagglutinin; Burroughs-Wellcome, London, England) was added at the optimal concentration of $1 \mu\text{g ml}^{-1}$. After 3 d, the cultures were pulsed with 1 μCi of ^3H -thymidine (6.7 Ci mmol^{-1} , New England Nuclear, Boston) for 16 h and collected on a filter paper using a multiple harvester (Mash II, Microbiological Assoc., Inc., Bethesda). Filter papers were counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton).

5. Response to con A: 4×10^5 purified PBL were added to microculture wells in 0.2 ml tissue medium as described above. Con A (Pharmacia) was added at an optimal concentration of $2.5 \mu\text{g ml}^{-1}$. ^3H -Thymidine uptake was measured as before.

6. Response to lipopolysaccharide: 5×10^5 purified PBL were added to microculture wells as above. LPS was added in a final concentration of $10 \mu\text{g ml}^{-1}$. After 2 d, ^3H -thymidine was added for 16 h and ^3H -thymidine uptake was measured as before.

* c.p.m. $\pm$ s.d.

† Mean $\pm$ s.e.

Table 2 Characteristics of peripheral blood lymphocytes of BALB/c mice after *in vivo* passage of BCL₁ cells*

	First passage	Second passage	Normal BALB/c mice
Absolute lymphocyte count mm ⁻³	128,000	352,000	5,200 ± 300†
% Ig-positive lymphocytes	85	90	30 ± 9‡
³ H-Thymidine uptake of unstimulated lymphocytes	251 ± 40‡	311 ± 86	87 ± 11
³ H-Thymidine uptake of PHA (1 γ ml ⁻¹) stimulated lymphocytes	1,811 ± 125	625 ± 160	82,531 ± 3,966
³ H-Thymidine uptake of unstimulated lymphocytes	252 ± 4	289 ± 1	115 ± 55
³ H-Thymidine uptake of con A (2.5 γ ml ⁻¹) stimulated lymphocytes	491 ± 56	380 ± 15	14,265 ± 850
³ H-Thymidine uptake of unstimulated lymphocytes	3,476 ± 504	5,057 ± 439	693 ± 70
³ H-Thymidine uptake of LPS (10 γ ml ⁻¹) stimulated lymphocytes	63,412 ± 5,539	67,392 ± 1,262	2,422 ± 15

* PBL obtained from sublethally irradiated (500 rad) recipients 12 weeks after intravenous injection of 5×10^6 spleen cells obtained from tumour-bearing donors. Technical data were described in legend to Table 1. Results of one representative experiment are shown.

† c.p.m. ± s.d.

‡ Mean ± s.e.

second passage was established by typing of the heavy and light chains of the surface Ig (μ , δ , λ) using immunofluorescent staining techniques (M. Knapp, S. Slavin, P. Jones, S. Black and S. Strober, in preparation).

An unexpected feature of the PBL of the BCL₁-bearing animals is the capacity to respond supranormally to LPS *in vitro*. It is unlikely that this response is produced by residual normal B lymphocytes in the PBL, since they are present in such small numbers. Investigation of the *in vitro* functional and cell surface characteristics of the BCL₁ cells in the blood and the solid lymphoid tissues is continuing, to try to clarify the relationship between cell-surface markers, cell maturity and functional characteristics. In conclusion, the BCL₁ line may provide a useful model for the study of human CLL, as well as normal and neoplastic B-lymphocyte biology.

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A new erythroid cell line induced by Rauscher murine leukaemia virus

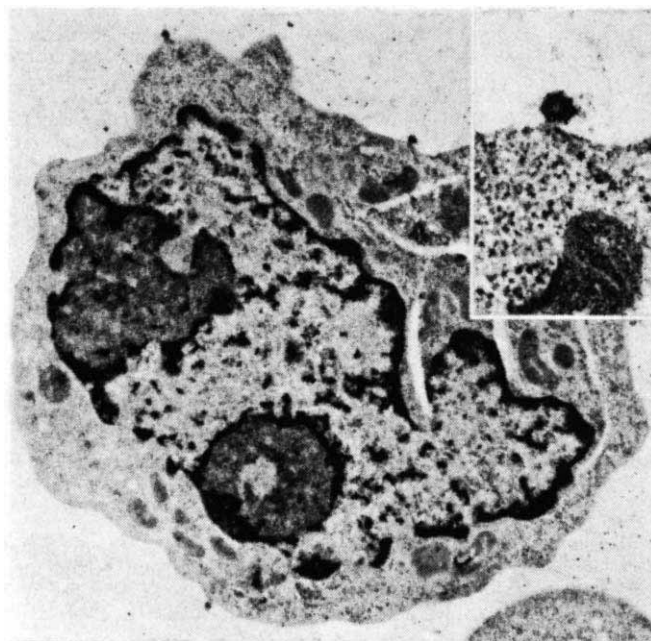
INOCULATION of BALB/c mice with high doses of Rauscher murine leukaemia virus (R-MuLV) results in rapid spleen enlargement due to the accumulation of immature erythroid cells, which subsequently infiltrate other haemopoietic organs^{1–3}. If low doses of virus are injected, the animals overcome the initial erythroid phase of the disease, but usually die later of a lymphatic leukaemia. In newborn C57/Black mice, R-MuLV induces lymphomas^{4,5}, whereas in (C57/Bl × DBA) F₁, various types of reticulum cell sarcomas are formed⁶. Attempts to establish permanent cell lines from these reticulum cell sarcomas were successful, but similar attempts with leukaemic spleen or liver tissue

failed repeatedly in BALB/c mice⁴. Instead, the blast cells matured into haemoglobin-containing cells *in vitro*⁷. We report here the induction of tumours in DBA mice using syngeneic transplants of R-MuLV-infected animals in the terminal phase of erythroid leukaemia. From this tumour, a new permanent cell line could be established, which could be induced to synthesise haemoglobin *in vitro*.

The tumours were induced by subcutaneous transplantation of liver fragments and could be transplanted serially both in solid and in ascites form. (DBA × C57Bl) and (DBA × BALB/c) F₁ crosses were also successful hosts for tumour transplantation, whereas transplantation failed in BALB/c mice.

The transplantable tumours were grown in Dulbecco modified Eagle's medium supplied with 20% foetal calf serum in 5% CO₂ at 37 °C. The homogeneous cell population morphologically resembled large immature blasts in the solid tumour and after prolonged culture *in vitro*. Ultrastructurally, dispersed chromatin with prominent

Fig. 1 Electron microscopy of cultured DBA erythroleukaemic tumour cells, 10th passage (magnification $\times 23,000$). Cells were harvested by low speed centrifugation, washed in Hank's balanced salt solution, and fixed at 4 °C for 2 h in 4% glutaraldehyde in 0.1 M cacodylate buffer – 0.1 M sucrose (pH 7.2). After washing in cacodylate buffer, the cells were resuspended in 1% OsO₄, and after repeated washings an equal volume of 2% liquid agar at 50 °C was added. The cells pelleted in agar were dehydrated in acetone and embedded in Epon-C. Polymerisation took place by incubation for 16 h at 37 °C followed by 25 h at 60 °C. Inset: budding virus at plasma membrane (magnification $\times 89,000$).



nucleoli was observed in the nucleus. The cytoplasm showed sparse cell organelles, a high number of free ribosomes and only a few polyribosomes (Fig. 1). Budding C-type virus particles were also observed (Fig. 1, inset).

The culture fluid was tested for infectivity on primary embryonic BALB/c fibroblasts. When infected fibroblasts were co-cultivated with XC-cells for 4 d according to Klement *et al.*⁸, many syncytia were observed. After pulse-labelling of 2×10^7 cells in 10 ml medium with $125 \mu\text{Ci}$ ^3H -uridine per ml (specific activity 15 Ci mmol^{-1}), 60S RNA, characteristic of oncornaviruses, could be extracted from 7th passage, but not from 14–16th passage culture fluids. These results indicate that the virus production decreased during the later passages.

To determine whether the solid tumour was a reticulum cell sarcoma, reticulin staining was carried out, but no reticulin fibres were histologically detectable. The cultured tumour cells did not show any phagocytic activity for latex or carbon particles. Treatment of the cells with fluorescent anti- θ -serum or anti-immunoglobulin showed no binding to the cell surface. These results suggest that it is unlikely that lymphoid cells are present among the tumour cells. The cultured tumour cells did not show any activity in the spleen colony forming assay⁹, nor were myeloid colonies formed in the soft agar culture method¹⁰. These data indicate that no haemopoietic stem cells or myeloid precursor cells were present amongst the tumour cells. Only undifferentiated tumour colonies were formed in the spleen.

The most important feature of the cells, however, was their erythroid nature. Evidence was derived from plasma clot cultures¹¹ in which 4% of the colonies became benzidine positive. Erythropoietin (Epo) alone increased the size of the colonies and induced erythroid differentiation (Fig. 2). After 4 d culture, about 35% of the colonies contained both benzidine positive and negative cells. Addition of 1.0% DMSO resulted in the formation of smaller benzidine positive colonies which increased to 25% of the total number of colonies within 3 d of culture, and to 88% within 6 d (data not shown), similar to Friend cells^{12–14}.

The largest stimulation was observed when DMSO and Epo were added simultaneously. Within 4 d, the percentage of large benzidine positive colonies increased to 93%, an increase dependent on the concentration of Epo. A fivefold increase in the number of positive colonies was observed in the DMSO + Epo-treated cells compared to the cells treated only with DMSO, and a 25-fold increase compared to the untreated control cultures. Apparently, Epo stimulates proliferation in these cells before the onset of haemoglobin synthesis, as reported for normal erythroid precursor cells¹⁵.

Haem and haemoglobin synthesis were analysed quantitatively by pulse-labelling with ^{59}Fe after treatment of the cells with DMSO and Epo for 72 h. The labelled haemoglobins were separated from labelled haem- and other Fe-containing proteins by chromatography¹⁶. The results (Table 1) show that in the untreated cells the haemoglobin/(haem) proteins (Hb/X) ratio was about 0.05, whereas 1% DMSO treatment caused a threefold increase of this ratio, while the addition of Epo also had a stimulating effect. The largest incorporation of ^{59}Fe was obtained by the simultaneous treatment of the cells with DMSO and Epo, producing an eightfold increase in the Hb/X ratio. When the total amount of haem was extracted with ethylacetate, the increase of ^{59}Fe was even more pronounced; a 50-fold increase in haem synthesis was measured in cells treated with DMSO and Epo for 4 d. When cells were first treated with DMSO or Epo alone for 2 d and then cultured in the presence of both DMSO and Epo, the treatment with DMSO had by far the largest stimulating effect (Table 2). This indicates that the cells become more sensitive to Epo by DMSO pre-treatment, as reported for normal erythroid cells¹⁷.

The results show that the R-MuLV complex is able to transform cells *in vivo*, from which a stable proerythro-

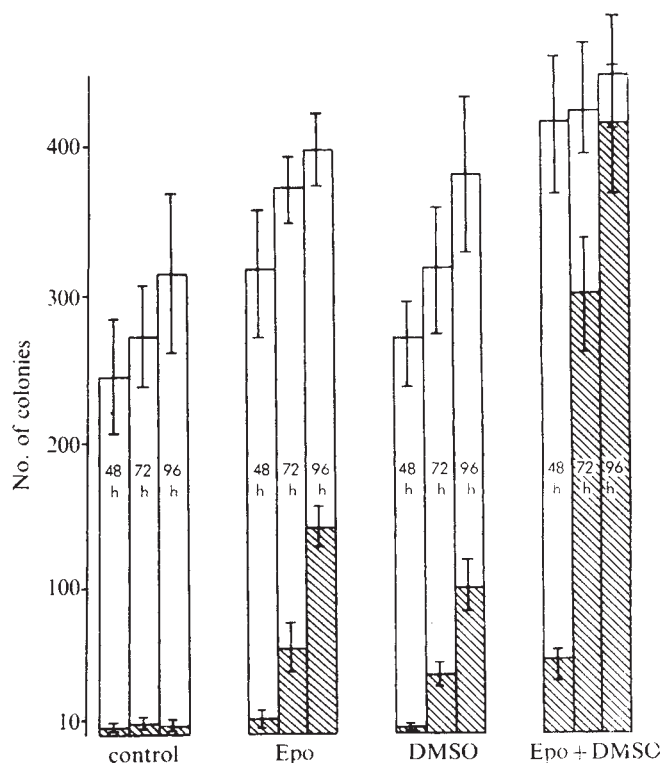


Fig. 2 Induction of haemoglobin synthesis in DBA erythroleukaemic cells cultured in plasma clot system¹¹. 2×10^3 cells were seeded per clot and the number of total and of benzidine positive colonies were scored over 48–96 h after culture in the presence of DMSO (1%) and/or Epo (0.25 units ml^{-1}). [—], total no. of colonies; [■], benzidine positive colonies; vertical bars indicate standard deviations ($n = 10$ clots).

blast-like cell line can be established. This cell line can be induced to synthesise haemoglobin in the presence of DMSO and Epo. An enhanced haem synthesis is also reported in one of four cell lines obtained from R-MuLV-induced reticulum cell sarcomas¹⁸, but it is not known whether or not haemoglobin synthesis takes place in this cell line.

The importance of DMSO is often stressed as the inducer of haemoglobin synthesis in Friend cells, these being

Table 1 Incorporation of ^{59}Fe in haemoglobin and other Fe-containing proteins of cultured Rauscher erythroleukaemic cells

	Control	DMSO (1%)	Epo (0.25 U ml^{-1})	Epo + DMSO
(Haem)protein-X-fraction (c.p.m. per 10^6 cells)	20,650	14,450	36,850	45,630
Haemoglobin (c.p.m. per 10^6 cells)	11,160	2,410	3,540	20,500
Ratio Hb/X fraction	0.056	0.167	0.096	0.450

3×10^6 cells per ml were cultured in triplicate in 5 cm Petri dishes for 72 h with erythropoietin (Epo) (Connaught) and/or DMSO (BDH). Each dish was labelled with $1.5 \mu\text{Ci}$ of $^{59}\text{Fe}(\text{m})\text{citrate}$ (S.A. 15 Ci g^{-1}) for 24 h before collection. As carrier haemoglobin, 100 μl mouse blood was used and the cells were washed 3 times in isotonic phosphate-buffered saline. The cells were lysed in one-third volume of 1% Nonidet. Immediately afterwards the haemoglobin was stabilised with CO. Subsequently the solution was dialysed for 18 h against 0.01 M phosphate buffer (pH 6.9). After dialysis, 70 mg protein was first chromatographed on carboxymethyl cellulose with 0.01 M phosphate buffer (pH 6.9). The void volume contained the non-haemoglobin haem and other Fe-containing proteins (X-fraction). The haemoglobins were eluted with 0.01 M phosphate buffer (pH 8.9) (ref. 16). Radioactivity was determined in a γ counter. The median values are presented.

Table 2 Incorporation of ^{59}Fe in haem of cultured Rauscher erythroleukaemic cells

Culture period (d)	Control	DMSO	Epo (0.25 U $\cdot$ ml)	Epo + DMSO	Epo followed by DMSO	DMSO followed by DMSO
3	620	1,580	1,270	5,320	—	—
4	670	4,760	1,310	31,630	5,990	25,180

2×10^6 cells were cultured in triplicate. Each dish was labelled with $1.5 \mu\text{Ci}$ of ^{59}Fe -(III)-citrate for 24 h before collection. Haem was extracted with glacial acetic acid/ethylacetate (1:3) and counted in a γ counter. The median values are presented (CPM/ 10^5 cells).

independent of and insensitive to Epo for haemoglobin synthesis^{19–24}. Epo is reported to stimulate haemoglobin synthesis only after DMSO treatment^{12,25}. No increase in haemoglobin synthesis was observed when the cells were simultaneously treated with DMSO and Epo.

Controversy exists about the regulation of the level of Epo in Rauscher virus infected animals^{26,27}. When infected mice are made anaemic by phenylhydrazine injection, a compensatory reticulocyte production takes place, just as seen after injection of Epo²⁸. It seems likely, therefore, that DMSO potentiates erythropoietin sensitivity in erythroid cells transformed by the Rauscher virus complex.

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Is protein synthesis necessary for the commitment of lymphocyte transformation?

WHEN resting lymphocytes are exposed to concanavalin A (con A) they are stimulated to enter the mitotic cell cycle^{1,2}. As the cells transform, protein synthesis increases markedly prior to DNA replication and they increase in mass some threefold (blast formation)^{3,4}. That protein synthesis is necessary for lymphocyte transformation has been verified here using anisomycin, an inhibitor of protein synthesis⁵. The mechanism of anisomycin action on protein synthesis is highly selective; it inhibits peptidyl transferase activity, and hence peptide bond formation, on cytoplasmic (80S) ribosomes. In this study the inhibition of lymphocyte transformation by anisomycin was found to be reversible and this provided a means of examining whether protein synthesis is necessary for the initial commitment of the cells into the mitotic cycle. The experiments described here were designed to test the possibility that cells exposed to con A become committed to transform, even though transformation itself is blocked.

Spleen cells from BALB/c mice (6–8-week-old males) were cultured in RPMI medium without serum (Fig. 1 legend). Mitogenesis was measured by the incorporation of ^3H -thymidine into cellular DNA during the period 48–50.5 h after exposure to con A; the stimulation index (SI) was calculated as c.p.m. con A-treated cultures/c.p.m. control cultures. The mitogenic response to con A peaked at $1 \mu\text{g ml}^{-1}$ and this concentration of con A was used in all the following experiments. Anisomycin at 3.8 nM had no effect on transformation whilst 10 nM anisomycin reduced the SI from 47 to 2 and at 38 nM anisomycin completely blocked con A-induced transformation (Fig. 1).

The inhibitory effect of anisomycin was shown to be reversible by removing the antibiotic after 24 h incubation and incubating the lymphocytes for a further 24 h in

Fig. 1 BALB/c spleen cells exposed to con A ($1 \mu\text{g ml}^{-1}$): dose-response curve showing inhibition of transformation by anisomycin in cells labelled for 48–50.5 h. Washed spleen cells from a single mouse were diluted to $5 \times 10^6 \text{ ml}^{-1}$ with RPMI plus 100 IU ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin and con A $1 \mu\text{g ml}^{-1}$. Control cultures contained no con A. Aliquots (100 μl) of these cells were dispensed into flat-bottomed wells of a microtitre tray and cultured in a humidified atmosphere of 10% CO_2 in air at 37°C . DNA synthesis was measured using standard techniques after labelling each culture with 2 μCi [^3H]thymidine (specific activity 5 Ci mmol^{-1}) for 2.5 h.

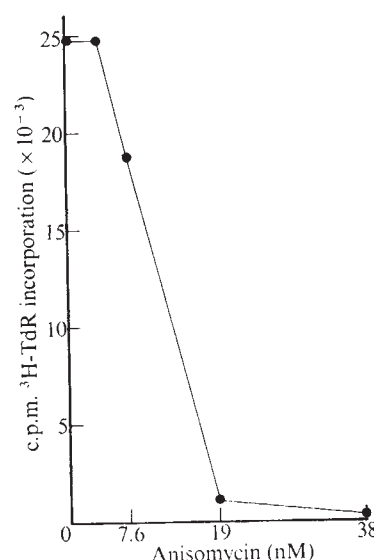


Table 1 Reversible inhibition of con A-induced transformation by anisomycin

Anisomycin concentration (nm)	³ H-TdR incorporation (c.p.m.) and SI Group		
	I	II	III
0	24,753 SI = 47	—	—
7.6	18,697 SI = 36	26,481 SI = 51	25,762 SI = 49
19	1,034 SI = 2	17,739 SI = 34	16,836 SI = 32
38	391 SI < 1	3,374 SI = 6	3,649 SI = 7

BALB/c spleen cells were cultured as described in the legend to Fig. 1. The reversibility of anisomycin inhibition was tested at 24 h by replacing the medium containing anisomycin plus con A ($1 \mu\text{g ml}^{-1}$) with fresh medium plus con A ($1 \mu\text{g ml}^{-1}$) (group II) or with fresh medium plus αMM (50 mM, group III). In group III the medium was replaced at 25 h with normal medium. Group I, cultures exposed to con A plus anisomycin continuously. Uptake of ³H-thymidine was measured at 48–50.5 h and each value represents the mean of five cultures.

medium plus con A (Table 1). The ability of the cells to transform following removal of both anisomycin and con A was determined by similar experiments except the fresh medium contained no con A. In addition α -methyl-D-mannoside (αMM) was used to displace any con A bound to the surface of the cells. (Exposure of cells preincubated with con A ($1 \mu\text{g ml}^{-1}$) to αMM (50 mM) for 1 h reduced bound con A to non-mitogenic levels: SI con A-treated cells=47, SI after removal of con A by αMM =1.5.) The ability of lymphocytes to transform following removal of both anisomycin and con A was similar to that observed when only the anisomycin was removed (Table 1).

The con A-induced transformation of splenic lymphocytes is clearly sensitive to very low concentrations of anisomycin and in a series of 16 experiments 38 nM anisomycin was sufficient to block transformation completely. These low concentrations of anisomycin blocked the increase in protein synthesis in con A-stimulated cells, as determined by measuring the incorporation of ³⁵S-methionine into proteins (in preparation).

It is well established that the rate of protein synthesis^{6,7} and cell protein content^{3,4} increase during lymphocyte transformation and the results presented here confirm that an increase in protein synthesis is essential for transformation. It is important to note that transformation was blocked by anisomycin concentrations which were not cytotoxic, as the inhibitory effect was fully reversible after removal of the antibiotic.

The observation that lymphocytes become independent of extracellular con A for transformation (after removal of both anisomycin and con A) is particularly interesting because it implies that commitment of lymphocytes to enter the mitotic cycle may be independent of protein synthesis. The cells become committed to transform within 24 h but it is unlikely that this long period is necessary for commitment since it has recently been shown that lymphocytes can become committed to transform during 3-h exposure to con A⁸. Thus it is possible that the signal(s) committing lymphocytes to transform may be stable or, alternatively, be continually produced in the presence of con A.

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A new tumour-derived transforming strain of Epstein-Barr virus

EPSTEIN-BARR VIRUS (EBV) has been associated with several diseases, including infectious mononucleosis (IM), Burkitt's lymphoma, and nasopharyngeal carcinoma. If EBV has an aetiological role in more than one of these diseases, explanations for pathogenic plurality must be sought. Host and environmental factors may influence the outcome of EBV infection, but recent descriptions of different EBV 'strains' have focused attention on the possibility that different virus strains may be associated with each of the above diseases^{1–3}. Although virus strains have not been differentiated by antigenic expression⁴, two major biological patterns have been delineated^{5–7} – strains capable of 'transforming' human cord blood lymphocytes to Epstein-Barr nuclear antigen (EBNA) positivity and 'immortalised' growth characteristics, and strains which lack this capability. In particular, the rare viral particles contained in throat washings from patients with active IM or Burkitt's lymphoma have such *in vitro* transforming capabilities^{8–10}. Until now, however, sufficient virus for biochemical study has been obtained from only two continuous lymphoblastoid cell lines. Fortunately, the viruses from these two lines have different biological properties. The B95-8 cell line produces a virus with transforming characteristics which was originally derived from the peripheral blood of a patient with transfusion-induced IM, and was passaged and maintained in lymphocytes of the cotton-topped marmoset¹¹. It is possible, however, that deletion or addition of nucleotide sequences has occurred as a result of the continued propagation of this virus in the cells of a subhuman primate. A second virus strain is produced by the P3HR1 cell line. This cell line was originally derived from the JIJoye Burkitt's lymphoma cell line by soft agar cloning¹². The virus produced by this cell line is generally incapable of *in vitro* 'transformation'^{5,6}, but will induce early antigen (EA) in the EBV genome-positive non-producing RAJI cell line¹³. EBV produced during early passage(s) of the original P3HR1 cell line had *in vitro* 'transforming' capability¹⁴, but subsequently lost this property. Studies by Zur Hausen¹⁵ have suggested that a P3HR1 cell line produces a mixture of EBNA transforming and EA-inducing virus. This raises the possibility of a mutation in the P3HR1 virus and concern regarding its use as a prototype or 'reference' strain of EBV. We report here the isolation of a new tumour-derived strain of EBV which has been propagated only in its cell of origin, and which manifests *in vitro* 'transforming' capability, making it a more representative 'wild-type' or putative tumour virus strain of EBV.

The B95-8 and P3HR1 virus strains have also been compared by restriction endonuclease mapping¹⁶ and by nucleic acid hybridisation¹⁷: B95-8 and P3HR1 DNA share 85–92% homologous sequences and it has been proposed that B95-8 represents a deletion mutant of P3HR1 (refs. 16, 17).

The new strain of EBV was isolated from a cell line (AG876) established from the splenic tumour of an 8-yr-old Ghanaian boy with Burkitt's lymphoma. Tumour tissue obtained during surgery was minced and the cells suspended in RPMI 1640 + 10% foetal calf serum (FCS, GIBCO). The cells continued to proliferate and have been maintained as a suspension culture in RPMI 1640 plus 10% heat-inactivated (56 °C, 30 min) FCS in 75-cm² plastic flasks at 37 °C. Almost all the cells contain EBNA, and 5–20% manifest the late viral product, cytoplasmic viral capsid

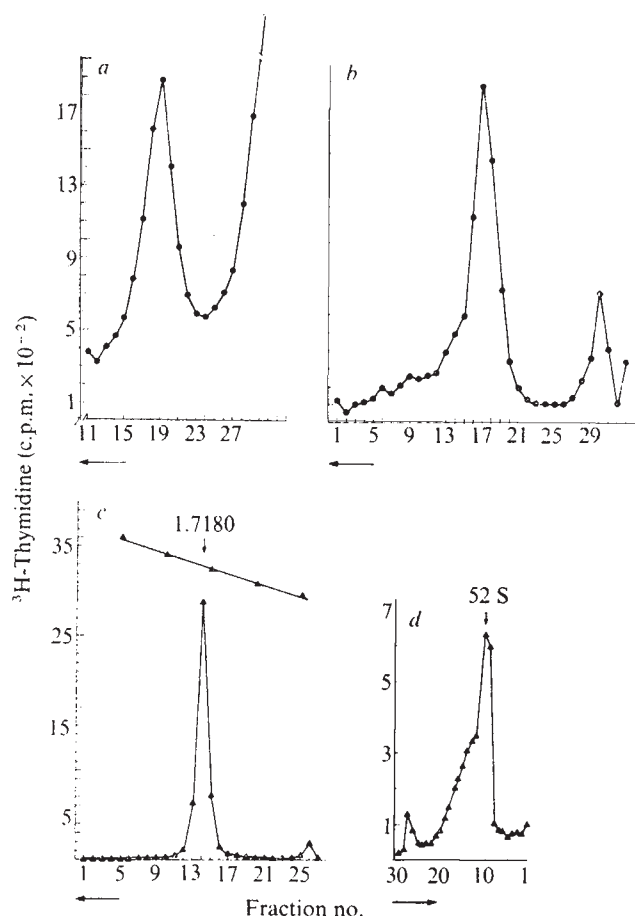


Fig. 1 Ultrafiltered supernatant virus concentrate was sonicated (three times for 10 s) using a sonifier cell disruptor (Bronson-Sonic Power Co) and then layered on to a Dextran (MW 10,000; Pharmacia) gradient (a) with a density range of 1.04–1.09 g cm⁻³, and centrifuged at 81,500g for 70 min at 4 °C. Aliquots of fractions of the gradient were counted in Aquasol (Packard) and the middle virus peak pooled, diluted threefold with virus buffer (VB) (0.01 M Tris, pH 7.6; 0.1 M NaCl) and pelleted at 113,000g for 1 h at 4 °C. The virus was again resuspended in VB, sonicated, and then layered on to a 30–60% (w/v) sucrose gradient (b) in TE buffer (0.01 M Tris, pH 7.6; 0.001 M EDTA), and centrifuged at 81,500g for 3 h at 4 °C. Fractions containing the virus were pooled, diluted threefold with TE buffer and pelleted. The caesium chloride gradient (c) was prepared from purified detergent lysed virus (2% Sarkosyl, 60 °C for 3 min), which had been extracted twice with phenol and with chloroform-isoamyl alcohol, and precipitated with ethanol. The gradient was centrifuged at 129,000g for 60 h at 20 °C and fractions were counted in Aquasol + 10% water. The ³H-thymidine peak had a density of 1.7180 g cm⁻³. Purified virus lysed with 2% Sarkosyl (60 °C for 3 min) was also layered on to a 10–30% sucrose gradient (d) in 0.1 M NaCl, 0.01 M Tris, pH 7.5, 0.01 M EDTA and spun at 198,000g for 3 h at 20 °C. Sedimentation markers included ¹⁴C-thymidine-labelled λ phage and SV40 form I DNAs (Bethesda Research Laboratory).

antigen (VCA). The AG876 virus has only been propagated in the cell line derived directly from this splenic tumour.

Cells to be used for virus production were cultured continuously for 14 d at 32 °C in 150-cm² plastic flasks (Corning). They were labelled on the 8th day of culture with ³H-thymidine (Amersham/Searle: 45 Ci mmol⁻¹) at a concentration of 5 μ Ci ml⁻¹. On the 14th day of culture, cells and supernatant were collected and the supernatant clarified of cellular debris by low speed centrifugation, and the resultant unconcentrated virus in the supernatant was assayed for transforming activity with Ficoll-Hypaque purified human umbilical cord leukocytes. Cord leukocytes (4×10^6 – 5×10^6) in 1 ml of RPMI 1640+20% FCS were incubated at 37 °C in 5% CO₂ atmosphere for 18 h. The cells were then pelleted, and resuspended in 0.2 ml of the clarified AG876

supernatant. After incubation for 90 min at 37 °C, the volume was adjusted to 1 ml with fresh RPMI 1640+20% FCS, and incubation continued at 37 °C for up to 6 weeks. Lymphocyte transformation was assayed by the appearance of cell aggregates and by increased metabolic activity manifested as increasing acidity in the culture medium. By this method, 0.2 ml of unconcentrated AG876 supernatant virus was found to contain $10^{4.5}$ 50% transforming units¹⁸. This same supernatant virus failed to induce EA production in RAJI cells. This biological pattern is, therefore, similar to that observed for B95-8 and unlike that of the P3HR1 virus strain⁵⁻⁷.

For biochemical analysis, the AG876 supernatant was first filtered through a 1- μ m Millipore filter (RATF 14200) and then concentrated by passage through a Millipore Pellican cassette system containing 4 sq. ft of filter with a nominal molecular weight cutoff of 1×10^6 – 3×10^6 . This approximately 20-fold concentrated supernatant was then pelleted at 113,000g for 1 h at 4 °C and resuspended in 3 ml of buffer (0.01 M Tris, pH 7.6; 0.001 M EDTA, 0.1 M NaCl). Subsequent virus purification steps included a dextran velocity gradient and isopycnic sedimentation in sucrose (Fig. 1a, b). Pelleted material from the middle radioactive peak in the isopycnic sucrose gradient was stained with 2% phosphotungstic acid in 0.4% aqueous sucrose, pH 6.0, and examined in a Siemens Elmiskop 1A electron microscope. Negatively stained herpes-like virus particles were seen. Purified virus was lysed with 2% Sarkosyl (Geigy; 60 °C for 3 min), and was found to contain 52S DNA when centrifuged in a 10–30% sucrose gradient (Fig. 1d). Viral DNA was purified by lysis in 2% Sarkosyl and 1% SDS (60 °C for 3 min and then 37 °C for 1 h), followed by two extractions with buffer (0.05 M Tris, pH 8.0; 0.01 M EDTA, 0.1 M NaCl) saturated distilled phenol, and two extractions

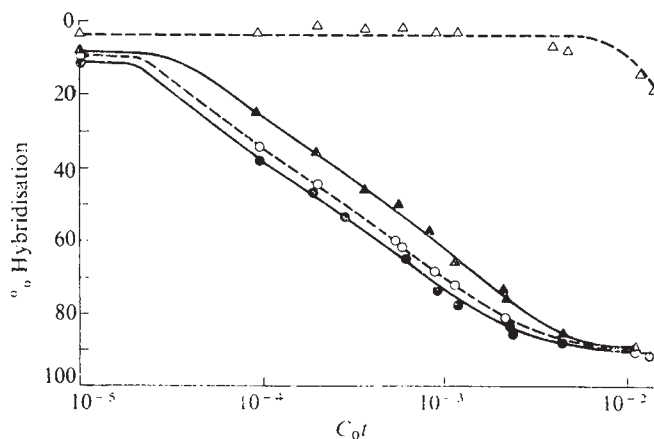


Fig. 2 Cellular DNA was extracted from cell pellets according to a method described previously¹⁹ which includes detergent lysis, enzymatic digestion of protein and RNA, phenol extraction, and ethanol precipitation. DNA with a ratio $A_{260}:A_{280}$ of 1.80–1.95 was then sheared and purified further as described previously. For hybridisation reactions, 7×10^{-2} μ g of ³H-AG876 viral DNA (specific activity $\sim 5 \times 10^4$ c.p.m. μ g⁻¹) was mixed with 5.6 mg ml⁻¹ of each sheared cold cellular DNA in a 'Reactival' (0.3 ml or 1.0 ml capacity). The mixtures were denatured in 0.12 M phosphate buffer (PB) by heating to 108 °C (in ethylene glycol) for 5 min, then adjusting to 0.48 M PB. Incubations were carried out at 65 °C. Sixty μ l samples were taken at different times and diluted to 2.0 ml with a final concentration of 0.14 M PB+0.4% SDS. Hybridisation was assayed by hydroxyapatite chromatography (Bio-Gel HTP, BioRad). Unhybridised molecules were eluted from the column with 8 ml 0.14 M PB+0.4% SDS and hybridised molecules with 8 ml of 0.32 M PB at 60 °C, to which 12 ml of Instagel (Packard) was added for liquid scintillation counting. ³H-AG876 viral probe compared with: ●, cold HR1 cell DNA; ○, a cold B958 cell DNA; △, cold AG876 cell DNA; □, human placental DNA. Data are presented as the percentage saturation of viral DNA as a function of C_0t (product of the viral DNA concentration in the reaction mixture and the duration of the hybridisation reaction, calculated as (nucleic acid absorbency at 260 nm $\times$ h of incubation $\div$ 2).

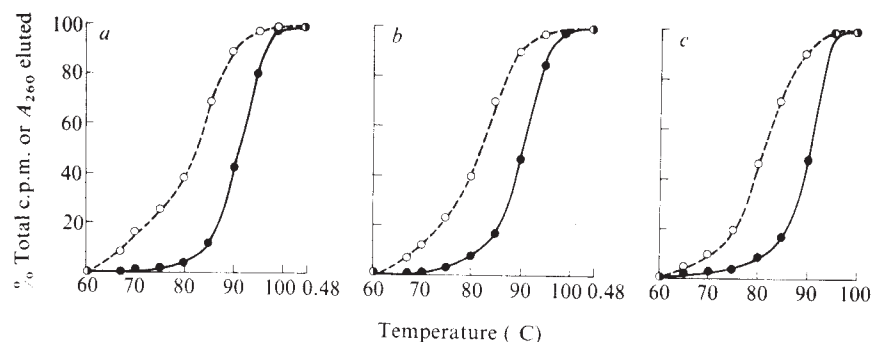


Fig. 3 Thermal elution profiles were assayed using aliquots of the hybridisation mixtures after 48 h of reaction. Each sample was diluted to 0.14 M PB+0.4% SDS and then passed over a hydroxyapatite column (60 °C). The fraction of molecules remaining single stranded was eluted with 0.14 M PB+0.4% SDS, and the temperature of the column was then raised in a series of 5 °C increments. After each temperature increment, the column was washed twice with 8 ml of 0.14 M PB+0.4% SDS. Each fraction was measured for absorbance at 260 nm to determine the percentage of hybridisation and the T_{e50} of cellular DNA hybrids. After addition of 12 ml of Instagel, the radioactivity and the melting profile of the viral DNA hybrids. The melting profile was determined by calculating the percentage of the total A_{260} units or c.p.m. which were bound to hydroxyapatite at 60 °C and which were eluted at each temperature. *a*, Hybrids formed between the ^3H -AG876 viral probe and cold AG876 cell DNA; 79.4% of the input A_{260} (○) and 96.8% of the input radioactivity (●) were bound to hydroxyapatite at 60 °C. *b*, Hybrids formed between the ^3H -AG876 viral probe and cold B958 cell DNA; 79.7% of the input A_{260} (○) and 93.5% of the input radioactivity (●) were bound to hydroxyapatite at 60 °C. *c*, Hybrids formed between the ^3H -AG876 viral probe and cold HR1 cell DNA; 79.5% of the input A_{260} (○) and 93.7% of the input radioactivity (●) were bound to hydroxyapatite at 60 °C.

activity in each fraction was determined in order to assay the extent of viral DNA hybridisation and the melting profile of the viral DNA hybrids. The melting profile was determined by calculating the percentage of the total A_{260} units or c.p.m. which were bound to hydroxyapatite at 60 °C and which were eluted at each temperature. *a*, Hybrids formed between the ^3H -AG876 viral probe and cold AG876 cell DNA; 79.4% of the input A_{260} (○) and 96.8% of the input radioactivity (●) were bound to hydroxyapatite at 60 °C. *b*, Hybrids formed between the ^3H -AG876 viral probe and cold B958 cell DNA; 79.7% of the input A_{260} (○) and 93.5% of the input radioactivity (●) were bound to hydroxyapatite at 60 °C. *c*, Hybrids formed between the ^3H -AG876 viral probe and cold HR1 cell DNA; 79.5% of the input A_{260} (○) and 93.7% of the input radioactivity (●) were bound to hydroxyapatite at 60 °C.

with equal volumes of chloroform-isoamyl alcohol (24:1 v/v). The aqueous phase was then adjusted to 0.2 M Na-acetate, precipitated with 2 vol. ethanol (−20 °C, overnight), pelleted at 109,000g for 30 min, resuspended in 0.01 M Tris, pH 7.8, 0.001 M EDTA, and dialysed against the same buffer for 48 h. DNA purified by this method had a ratio of A_{260} to A_{280} of 1.90. AG876 DNA centrifuged to equilibrium in CsCl banded at a density of 1.7180 g cm^{−3} (Fig. 1c), which is consistent with previous analyses of EBV DNA. The maximum yield of AG876 viral DNA obtained by us was 5 μg l^{−1} (measured spectrophotometrically, assuming 21 A_{260} units = 1 mg of DNA) of harvested supernatant. The specific activity was 50,000 c.p.m. μg^{−1}.

For nucleic acid hybridisation experiments, purified radiolabelled viral DNA was sheared twice by passage through a needle valve with a pressure drop of 40,000 p.s.i. using a French pressure cell (American Instrument Co). Sheared DNAs were filtered through a metrical GA-6 filter (cellulose acetate, Gelman), extracted with chloroform-isoamyl alcohol (24:1 v/v), and ethanol-precipitated as described above. Association kinetic experiments (Fig. 2) revealed 90% or greater hybridisation of the labelled AG876 viral DNA probe with its respective producer host cell DNA, as well as with the cellular DNAs from P3HR1 and B95-8 producer cell lines. Furthermore, there was virtually 100% homology in the kinetics of hybridisation of AG876 with P3HR1 and B95-8 DNAs, suggesting close similarity between the AG876 viral DNA and that of both P3HR1 and B95-8.

The thermal elution profiles of the hybrids formed between the viral ^3H DNA and the cellular DNAs (Fig. 3) showed the labelled AG876 viral DNA to be a homogeneous population distinct from the cell DNA. This AG876 viral DNA has a T_{e50} (midpoint of the thermal elution profile) of 91.5 °C compared with 81.5 °C for the cellular DNA. The thermal elution profiles of the AG876 viral probe with AG876, P3HR1 and B95-8 cell DNAs were similar.

This newly derived virus (AG876) seems to be a unique strain of EBV. Unlike B95-8, it was derived from a tumour, and has never been propagated outside of its human cell of origin. It is also distinguished from the only other tumour-derived strain available at present in sufficient quantities for biochemical studies (P3HR1), by its consistent *in vitro* transforming ability and lack of EA-inducing capacity. Nucleic acid hybridisation analysis has failed to reveal discernable differences between the AG876 and B95-8 strains within the sensitivities of this technique. Moreover, the AG876 does not seem to lack sequences when compared with P3HR1 DNA, as has been

reported for B95-8 (ref. 17), although the capability for *in vitro* transformation is maintained.

It seems, therefore, that the AG876 virus may be more representative of a 'wild-type' (or putative tumour virus) strain of EBV, than other available strains. Comparison of these and other strains that may be isolated should prove important in further elucidating the association of EBV with various diseases.

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Circulating immune complexes in infants fed on cow's milk

CIRCULATING antigen-antibody (AgAb) complexes are capable of damaging various tissues, for example, renal glomeruli, vessels and joints¹. In addition, immune complexes (IC) can interfere with cellular immunity and, to some extent, decrease the immune response²⁻⁴. We report here that all infants fed on cow's milk have circulating complexes containing bovine proteins as antigens and IgG antibodies of maternal origin. These data raise questions as to the possible involvement of such complexes in various disorders, the intestinal resorption of macromolecules, and the development of antibodies against food antigens.

IC were detected by their ability to inhibit the agglutinating activity of rheumatoid factor (RF) or purified human C1q, a constituent of the first factor of complement, towards particles coated with human IgG⁵. As RF, we used the suitably diluted (1/200) serum of a patient with rheumatoid arthritis.

The tests were performed on blood samples from 46 neonates, on their cord sera as well as on serum samples collected 6 d after birth. While their cord sera were negative, 24 had, on the sixth day, factors inhibiting RF and C1q at serum dilutions ranging from 1/2 to 1/8. Of these 24, only one was breast-fed. The others received either cow's milk alone ($N=11$) or as a supplement to breast-feeding ($N=12$). Of the 22 infants without IC, 21 were breast-fed and one received mixed food.

Large excess of antigen is known to reduce the inhibitory activity of immune complexes⁶. Hence, the addition of bovine milk proteins to these sera with inhibitory activity should decrease the reaction with RF or C1q, if the antigen of the immune complexes came from the milk. A 1% solution of the milk powder which was used for feeding the babies (Guigolac) was prepared in saline. One volume of this solution, which was itself devoid of any agglutinating or inhibitory activity, was incubated for 30 min at 37 °C and overnight at 4 °C with one volume of serum from each of two infants which inhibited RF and C1q up to 1/8 dilution. After addition of bovine milk, the sera completely lost their inhibitory activity. Controls were performed on two cord sera containing immune complexes (inhibition titres 1/8 toward RF and C1q) as a result of neonatal infections⁶, and allowing for the effect of dilutions on the inhibition titres, no significant changes were observed after addition of bovine milk to these sera. By showing that the immune complexes contained bovine milk proteins as antigen, we confirmed that the neonatal intestine is permeable to macromolecules⁷⁻⁹.

That the antibody involved in the complexes was Ig_G was suspected by exclusion of the other main Ig classes. RF is known to be poorly inhibited by polymerised IgM, and polymerised IgA does not inhibit the agglutinating activity of C1q⁵. As all positive sera reacted with both RF and C1q, it was unlikely that these Ig were involved in the immune complexes.

In the 6-d-old infants, most IgG antibodies are of maternal origin. Hence, the anti-milk antibody was presumably acquired through the placenta from the mother. If this hypothesis were true, the addition of mothers' sera to infants' sera should increase the proportion of antibody to antigen, and thus the size and inhibitory activity of the complexes¹. As sources of maternal immunoglobulins, we used cord sera. One volume of infant's serum was mixed with one volume of the serum from its own cord blood, and incubated at 37 °C for 30 min, and then at 4 °C overnight. This experiment was performed on sera from 43 infants whose cord sera were devoid of immune complexes. The addition of cord serum caused a significant increase

of the inhibitory activity (at least two dilution titres) in 17 sera. Of these, 13 came from infants receiving cow's milk or mixed feeding ($N=21$). Of the 26 sera in which no increase of the inhibitory activity was observed, 18 were from breast-fed infants ($N=22$). The correlation between the antigen-antibody reactions and the milk source was highly significant ($\chi^2 = 7.6$; $0.01 > P > 0.001$).

The existence of antibody against bovine milk in cord blood was confirmed by counter-electrophoresis using eight dilutions of Guigolac, from 0.08 to 10 mg ml⁻¹. The occurrence of IgG antibody against bovine casein in cord blood had already been reported¹⁰.

To check the frequency of anti-bovine milk antibody, we collected serum from 50 healthy members of our staff. All these sera gave a precipitin line against at least one dilution of Guigolac as did a commercial preparation of γ -globulins (165 mg ml⁻¹) (Institut Mérieux). To check that the precipitin line was really the result of an antigen-antibody reaction, F(ab')₂ fragments were prepared from γ -globulins by pepsin digestion¹¹. A solution of 10 mg ml⁻¹ F(ab')₂ fragments also gave a clear precipitin line.

Antibody activity against bovine milk has been detected in IgA from mother's milk^{12,13}. So, mixed feeding should prevent the resorption of cow's milk proteins. However, we did not find less circulating immune complexes in infants on mixed feeding.

Various disorders have been attributed to bottle feeding such as allergy and recurrent infections^{14,15}. By their interference with the immune response, immune complexes could be involved in the development of these disorders. A possible long-term consequence of the occurrence of circulating immune complexes during infancy is arteriosclerosis. In animals, serum sickness accelerates its development¹⁶⁻¹⁸, and coronary lesions are more common in people who were fed on cow's milk than in those who were breast-fed^{19,20}. By damaging the arterial endothelium, the immune complexes could facilitate the deposition of lipids and the formation of the atheroma plate²¹.

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Human pulmonary alveolar macrophages metabolise benzo(a)pyrene to proximate and ultimate mutagens

PULMONARY alveolar macrophages (PAM) phagocytose inhaled foreign particulates that reach the peripheral airways. Particulates engulfed by PAM are then transported by the mucociliary system up the respiratory tract past the bronchial epithelium to be expectorated and/or swallowed. Since these particulates may contain adsorbed chemical carcinogens, for example, those in tobacco smoke, and PAM have been shown to metabolise chemical carcinogens¹⁻³, PAM may interact with the bronchial epithelium in the metabolic activation of respiratory carcinogens. We report here the metabolic activation of benzo(a)pyrene (B(a)P) by PAM into both a promutagen ($\pm$)7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene; (7,8-diol; 50% (+)) and an ultimate mutagen(s).

For the detection of mutations, the cell-mediated mutagenesis system first described by Huberman and Sachs⁴ was modified by substituting, as the activating cell type, human PAM in place of Syrian golden hamster embryo cells. The indicator cells, Chinese hamster V-79 cells⁵, do not effectively metabolise B(a)P and its 7,8-diol into the ultimate mutagen(s)⁶. PAM were isolated from surgical and autopsy specimens of non-tumorous peripheral lung (patient 157, 62-yr-old male with epidermoid carcinoma of the lung; patient 161, 66-yr-old male with combined epidermoid-adenocarcinoma of the lung; patient 165, 16-yr-old male who died of head trauma) as described previously³. The culture medium was CMRL-1066 (Grand Island Biological, Grand Island, New York) supplemented with 10% fetal bovine serum (Irvine Scientific, Irvine, California), hydrocortisone hemisuccinate ($0.1 \mu\text{g ml}^{-1}$; Upjohn, Kalamazoo, Michigan), crystalline bovine insulin ($1 \mu\text{g ml}^{-1}$; Eli Lilly, Indianapolis, Illinois), β -retinyl acetate ($0.1 \mu\text{g ml}^{-1}$; Hoffmann-LaRoche, Nutley, New Jersey), penicillin G (100 U ml^{-1} ; Grand Island Biological) and streptomycin ($100 \mu\text{g ml}^{-1}$; Grand Island Biological). For the cell-mediated mutagenesis assay, 5×10^5 V-79 cells were added to each 100-mm culture dish (Falcon Plastics, Oxnard, California) containing PAM which had been seeded 7-8 d earlier. After cocultivation for 8 h, the medium was replaced with media containing

Fig. 1 Determination of optimal expression time for isolating O^r mutants, that is, the time following exposure of 10^5 V-79 cells, cocultivated with human PAM from patient 157, to either 7,8-diol $1 \mu\text{g ml}^{-1}$ ($\bullet$); $0.2 \mu\text{g ml}^{-1}$ ($\blacktriangle$) or B(a)P $1 \mu\text{g ml}^{-1}$ ($\circ$) until the addition of 1 mM ouabain. In the control cultures without human PAM, V-79 cells were incubated with either 7,8-diol $1 \mu\text{g ml}^{-1}$ ($\square$) or B(a)P $1 \mu\text{g ml}^{-1}$ ($\blacksquare$) until the addition of 1 mM ouabain.

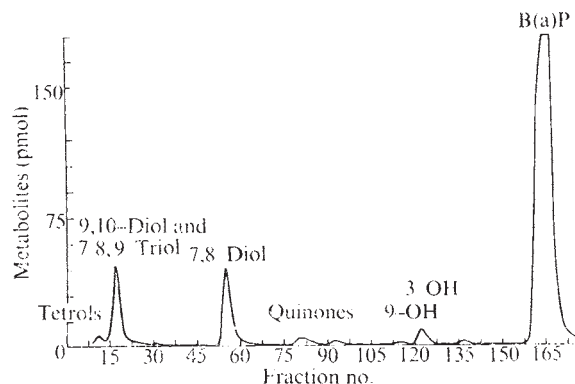
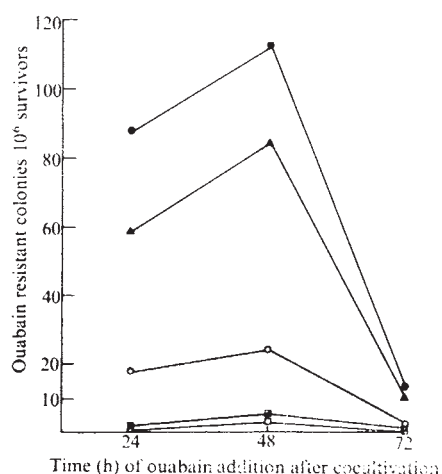


Fig. 2 Profile of B(a)P metabolites isolated from the culture medium of human PAM (patient 157) incubated with B(a)P. B(a)P metabolites were separated by high pressure liquid chromatography⁸. Nomenclature used for B(a)P metabolites has been reported¹³.

either B(a)P, B(e)P or 7,8-diol. The media were removed for determination of B(a)P metabolites using high pressure liquid chromatography^{7,8} and replaced with fresh B(a)P-containing medium every 24 h during the next 3 d. Similarly, the media containing either 7,8-diol or B(e)P was replaced every 24 h. Seventy hours after the treatment was initiated, the cocultivated cells were dispersed with 0.05% trypsin-EDTA solution (Grand Island Biological) and counted with a Coulter Model ZB counter (Coulter Electronics, Hialeah, Florida). The number of V-79 cells per dish was calculated by subtracting the number of macrophages in a control dish from the total cells in the dishes containing both V-79 cells and macrophages. The accuracy of this method for determining the number of macrophages was confirmed by visual cell counting based on differential cell size as described by Huberman and Sachs⁴. Approximately 10-20% of the cocultivated cells were PAM. The dispersed V-79 cells were seeded at 10^5 cells per 60-mm tissue culture dish (12-20 dishes) for selection of ouabain-resistant (O^r) mutants and at 100 cells per dish (5 dishes) for determination of cloning efficiency⁶. The selective medium containing 1 mM ouabain was added to the dishes for determining mutation frequency 24, 48 or 72 h later; the optimal expression time, that is, the time following exposure to either B(a)P or 7,8-diol until the addition of 1 mM ouabain for isolating O^r mutant V-79 cells, was 48 h (Fig. 1). After 2 weeks, the colonies were fixed with absolute alcohol and stained with

Table 1 Relationship between O^r mutation frequency of V-79 cells and number of cocultivated PAM.

Agent	Group*	Number of PAM	Cloning Efficiency (% control)	Mutation frequency O^r mutants/total dishes	O^r mutants/ 10^6 survivors
Control	—	—	100	6/20	4
B(a)P	C	—	66	2/17	3
	E	1.8×10^5	56	5/19	7
	E	3.1×10^5	50	7/18	10
B(e)P	C	—	92	5/15	5
	E	1.8×10^5	101	3/20	2
	E	3.1×10^5	94	6/17	5
7,8-Diol	C	—	72	5/20	4
	E	1.8×10^5	59	18/19	20
	E	3.1×10^5	40	15/13	38

*C, Control (V-79 cells incubated with B(e)P, B(a)P or 7,8-diol and without human PAM); E, experimental (V-79 cells incubated with either B(e)P, B(a)P or 7,8-diol and with macrophages).

PAM (patient 161) were cultured for 7-8 d and then 10^5 V-79 cells as well as B(a)P, B(e)P or 7,8-diol (each at $1 \mu\text{g ml}^{-1}$), were added per dish.

Cloning efficiency compared with that of V-79 cells incubated without both PAM and chemical agents (absolute value was 78.4%).

Table 2 Effect of dose of B(a)P and 7,8-diol on O^r mutation frequency in V-79 cells mediated by PAM

Agent	Group*	Dose ($\mu\text{g ml}^{-1}$)	Cloning Efficiency (% control)	O^r mutants/total dishes	O^r mutants/ 10^6 survivors	O^r mutants/ 10^6 survivors/ 10^6 PAM
Control		—	100	2/16	1	—
7,8-diol	C	1.0	97	6/13	3	—
	E	0.1	101	16/15	15	10
	E	0.5	91	71/15	70	46
	E	1.0	92	92/17	110	73
B(a)P	C	2.0	109	5/17	3	—
	E	0.2	87	3/14	2	2
	E	1.0	65	11/20	7	5
	E	2.0	60	7/12	7	5

*C, Control (V-79 cells incubated with either 7,8-diol or B(a)P and without human PAM); E, experimental (V-79 cells incubated with either 7,8-diol or B(a)P and with human PAM (patient 165). Cloning efficiency compared with that observed when V-79 cells were incubated without both chemical agent and PAM; the absolute cloning efficiency for V-79 cells cultivated without both PAM and chemical agents was 81.2%.

Geimsa for detection of O^r mutants. The O^r mutants have: the karyotype of V-79 cells; a stable phenotype for O^r after more than 20 cell divisions in medium without ouabain; and functional ATPase activity, as the rate of ^{86}Rb uptake in the presence of 1 mM ouabain is as expected (ref. 9 and Hsu *et al.*, unpublished).

The mutation frequencies for O^r in V-79 cells co-cultivated with PAM and B(a)P, B(e)P, or 7,8-diol are shown in Table 1. The mutation frequency is directly dependent on the number of PAM added to the V-79 cells in medium containing either B(a)P or 7,8-diol. B(e)P, the non-carcinogenic analogue of B(a)P, was not mutagenic. Cocultivation of V-79 cells with PAM but without B(a)P, B(e)P or 7,8-diol did not alter the O^r spontaneous mutation frequency of V-79 cells (less than two O^r mutants/ 10^6 surviving V-79 cells). An increase in concentration of either B(a)P or 7,8-diol enhanced O^r mutation frequencies (Table 2). Compared with B(a)P, 7,8-diol was a more potent promutagen. PAM also metabolised B(a)P to 7,8-diol which was released by PAM into the culture medium (Fig. 2)¹.

The clearance of inhaled particulates with adsorbed chemical carcinogens may be impaired by tobacco smoke, sulphur dioxide, anticholinergic drugs, chronic bronchitis and respiratory infections¹⁰. These particulates may also have a prolonged residence time in areas of squamous metaplasia because ciliary activity is interrupted in these areas. PAM phagocytose particulates and are also considered to play an important role as a host defence against inhaled foreign material¹¹. The data presented here suggest that they can also metabolise B(a)P to proximate and ultimate mutagens, which are then released into the extracellular space. The proximate mutagenic and carcinogenic form of B(a)P, 7,8-diol (ref. 12), can enter into cultured bronchial explants from the medium and has been shown to be more effectively metabolised and bound to DNA in the bronchial mucosa than the 4,5-diol, 9-10-diol and the parent compound, B(a)P (ref. 13). The intimate contact between PAM and bronchial epithelium during clearance by the mucociliary transport of particulates containing chemical carcinogens within PAM suggests the possibility that PAM and bronchial epithelium may act in concert to metabolically activate chemical carcinogens during bronchogenic carcinogenesis.

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Enhancement of carcinogenesis by prostaglandins

INCREASED amounts of prostaglandins, especially prostaglandin (PG) E_2 (PGE_2), have been detected in human cancer tissue as well as in cultured mouse fibrosarcoma cells¹. Elevated prostaglandin synthetase activity has also been found in microsomal fractions of transformed cells from methylcholanthrene-treated mice². In investigations of the role of PGE_2 and $\text{PGF}_{2\alpha}$ on skin tumours induced by 3-methylcholanthrene (MCA) in mice, I report here evidence that these prostaglandins can act as cocarcinogens.

The experiments were carried out on male albino Swiss mice weighing between 20 and 25 g. They were divided into six groups of 20 mice as follows: (1) mice which received only the diluent and served as controls; (2) mice treated topically with 0.2 ml of a 0.4% acetone solution of MCA in a marked region of the shaved dorsal skin, three times a week for 2 months; (3) mice treated with MCA as above and injected intramuscularly (i.m.) concomitantly with 10 μg of PGE_2 three times weekly for 2 months; (4) mice treated with MCA and injected i.m. concomitantly with 10 μg $\text{PGF}_{2\alpha}$ three times weekly for 2 months. Groups 5 and 6 were treated only with PGE_2 and $\text{PGF}_{2\alpha}$, respectively, as above. At the end of 2 months and 2 h before killing, 5 mice from each group received i.m. an injection of 10 μCi per g body weight ^3H -thymidine, for the study of DNA synthesis; another 5 mice from each group were injected i.m. with 10 μCi per g body wt ^3H -uridine, for the study of RNA

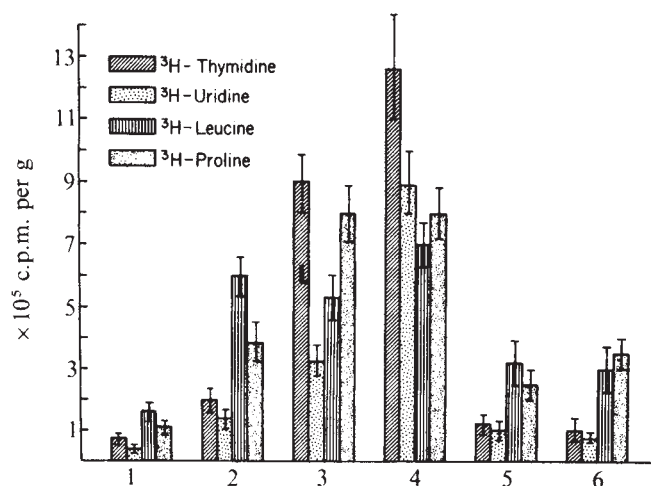


Fig. 1 Radioisotope incorporation at 2 h in the control mouse epidermis (1), following MCA (2), MCA + PGE₂ (3), MCA + PGF_{2α} (4), PGE₂ (5) and PGF_{2α} (6). The vertical bars at the top of each column represent the standard errors of the mean (mean ± s.e.).

synthesis; 5 other mice from each group received 10 μ Ci per g body wt ³H-leucine, for the study of protein synthesis, and the last 5 mice from each experimental group were injected i.m. with 10 μ Ci per g body wt ³H-proline, for the study of collagen synthesis. The period of 2 h before death was selected for the isotope studies, because previous experiments had shown that PGs exert their maximum effects on cell structure and metabolism in that time³. All radioisotopes were injected at the same time as PG and MCA administration. Radioactivity measurements were made using a nuclear liquid scintillation counting system (efficiency 40%), using (³H) as internal standard; the skin specimens were trimmed, the subcutaneous fat and dermis were removed and the epidermis was homogenised with a Potter-Elvehjem tissue grinder and transferred to vials with scintillation fluid. Results were expressed as counts per minute (c.p.m.) and per g of homogenates (mean ± s.e.). For light microscopy and autoradiography, the specimens were fixed in Bouin's fluid, dehydrated and embedded in paraplast; sections 5 μ m thick were stained with haematoxylin and eosin and examined under a light microscope. For autoradiography alone, the sections were covered with Ilford Nuclear Emulsion K₃ (diluted with distilled water 1:1) for 7 d, after which they were developed in D₁₉, fixed and washed, and stained with haematoxylin and eosin.

Multiple and large tumours, sometimes necrotic and haemorrhagic in the centre, occurred after 2 months in almost 90% of both the MCA plus PGF_{2α}- and MCA plus PGE₂-treated mice at the treatment site. No tumours were observed in MCA-treated, PG-treated or in control mice (Table 1) and no toxic or other adverse reactions occurred. Investigations with radioisotopes revealed a marked increase (15–20-fold) of ³H-thymidine, followed by ³H-uridine, ³H-proline and ³H-leucine in the homogenised material from tumours of MCA plus PGF_{2α} and MCA

plus PGE₂-treated mice, compared with those from MCA-treated, PG-treated or control epidermis (Fig. 1).

Histopathological examination showed a characteristic pattern of epidermal cells in control mouse skin (Fig. 2a). Epidermal cell hyperplasia with papillary projections protruding in the dermis, are visible in MCA-treated mice alone (Fig. 2b). Several invading epidermal masses in the dermis with a tendency to horn pearl formation are predominant in MCA plus PGF_{2α}-treated mice. These are squamous cell carcinomas (Fig. 2c) and they occurred in almost 90% of MCA plus PGF_{2α}-treated mice (see Table 1). At higher magnification, the tumour masses are composed of neoplastic squamous cells arranged in a palisading pattern. A mild dermal infiltration always accompanied the tumours. Few papillomas were seen in MCA plus PGE₂-treated mice and only a moderate epidermal hyperplasia occurred in PG-treated mice. Light microscopic autoradiography revealed an increased (5–6-fold) distribution of the heavily ³H-thymidine labelled nuclei in the neoplastic cells following MCA plus PGF_{2α} administration, compared with that seen in controls or in only MCA-treated mice (Fig. 2d).

The present findings demonstrate that PGF_{2α} and PGE₂ enhanced the transformation of hyperplastic epidermal cells of MCA-treated mice to neoplastic cells and thus markedly shortened the latency period to tumour formation, in a manner similar to that of cocarcinogens⁴. The investigations using MCA application revealed that the tumours occurred only after 4 months, and mostly between 5 and 6 months; the PGs therefore decreased (or shortened) the latent period of tumour induction (2 months) by at least 65%. It is possible that PGs which are hormone-like substances, enhance tumour formation, as do other hormones such as oestrogens, prolactin and thyroid stimulating hormone (TSH) (ref. 5), all of which stimulate mammary and thyroid hyperplasia of mammary and thyroid tumours. These agents are not carcinogenic by themselves; PGs administered alone for the same period (groups 5 and 6) induced only a moderate epidermal cell hyperplasia, but their effects are similar to those of other cocarcinogens. It has been suggested that naturally occurring prostaglandin agonists include some cathartics (rhein, emodin) and the cocarcinogens anthralin and phorbol myristate acetate, and thus that PGs themselves might be cocarcinogens⁶. PG antagonists exhibit an anti-tumour activity. Human malignant breast tumours contain and synthesise more PG-like materials than normal breast tissue from the same patients or than do benign tumours⁷. In patients with breast carcinomas, osteolytic activity and bone metastases, a high PG activity (PGF_{2α}, PGE₂) has been detected *in vitro*⁸. These findings suggest that PGF_{2α} and PGE₂ are important in the development of primary tumours and that they can influence the evolution of primary tumour metastases. PGF_{2α} added to Swiss mouse fibroblast cultures initiates DNA synthesis and cell proliferation. Insulin potentiates the effect of PGF_{2α} and DNA synthesis and cell division⁹. The present experiments also demonstrated that PGF_{2α} and PGE₂ markedly stimulate the DNA, RNA and protein synthesis in hyperplastic epidermal cells following MCA application. This could explain the enhancement of cutaneous carcinogenesis and shortening of the latent period. Electron microscopy has shown that during the latent period only preneoplastic changes resembling those of Bowen's disease are present in rat epidermis¹⁰. Thus, the present

Table 1 The incidence of skin tumours in mice following MCA and prostaglandin (PGE₂ and PGF_{2α}) administration

Group	Treatment	Time (months)	No. of mice	Epithelial hyperplasia	Papillomas	Carcinomas	% of Tumours
1	Controls + diluent	2	20	0	0	0	
2	MCA + diluent	2	20	20	0	0	
3	MCA + PGE ₂	2	20	2	2	16	90%†
4	MCA + PGF _{2α}	2	20	0	1	19	100%†
5	PGE ₂	2	20	18*	0	0	
6	PGF _{2α}	2	20	18*	0	0	

The data presented are based on counts of tumours visible to the naked eye, as well as on diagnosis made by light microscopy.

*Moderate epidermal hyperplasia.

†Papillomas and carcinomas.

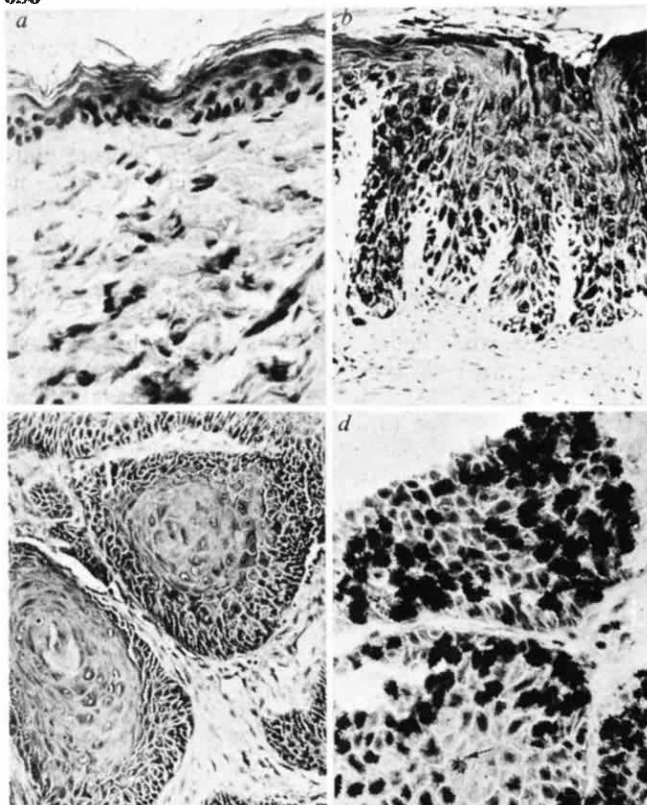


Fig. 2 *a*, Control mouse skin (haematoxylin and eosin, $\times 200$); *b*, MCA-treated mouse skin. Epidermal cell hyperplasia with papillary projections in the dermis (haematoxylin and eosin, $\times 200$); *c*, MCA + $\text{PGF}_{2\alpha}$ -treated mouse. Large and invading tumour masses with a tendency to horn pearl formation are predominant (squamous cell carcinoma); mild dermal infiltration. (haematoxylin and eosin $\times 200$); *d*, light microscopic autoradiogram of a MCA + $\text{PGF}_{2\alpha}$ -treated mouse showing a heavy autoradiographic reaction of the neoplastic cells in a squamous cell carcinoma. Some dividing cells and their chromosomes are labelled with ^3H -thymidine (arrow) (haematoxylin and eosin $\times 400$).

findings indicate that PGs can act as cocarcinogens and have important implications in cutaneous carcinogenesis.

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Enhanced agglutination of all the erythrocytes when only half are trypsinised

Enhanced lectin-mediated agglutination following alteration of cells by transformation or protease treatment is well documented^{1,2}. However, the mechanism underlying these observations remains obscure. Recent evidence suggests that enhanced agglutinability of erythrocytes treated with proteolytic enzymes, neuraminidase or both, may be the result of reduced surface charge and/or loss of sterically hindering peptides and glycopeptides³. We wondered whether the alteration in a part of the cell population would be sufficient to enhance the agglutinability of the entire cell population with concanavalin A (con A) and soybean agglutinin (SBA).

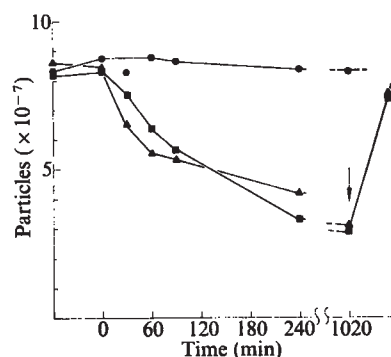
We report here that the extent, and to a slightly lesser degree, the rate of lectin-mediated agglutination of untrypsinised erythrocytes mixed with equal volumes of trypsinised erythrocytes closely parallel the values obtained for trypsinised cells alone. We conclude that the proteolytic alteration of only some of the erythrocytes is sufficient to promote enhanced lectin-mediated agglutination of all cells when mixed with equal numbers of native cells. A similar phenomenon holds true for native cells when mixed with neuraminidase-treated cells.

Human erythrocytes were obtained from fresh blood. After removing serum and buffy coat, erythrocytes were washed four times with 10-fold volumes of phosphate-buffered saline (PBS), pH 7.4. Following trypsinisation (0.1 mg ml^{-1} , Sigma) of 4% (v/v) erythrocyte suspensions in PBS for 60 min at 37°C , the cells were washed three times in PBS with or without 0.1 mg ml^{-1} soybean trypsin inhibitor (Sigma) in the first wash. Some cells were treated with trypsin in the presence of trypsin inhibitor in order to test for effects not attributed to proteolytic activity. Residual tryptic activity in the final washes of normal, trypsinised cells, and trypsinised cells washed with inhibitor, were twice tested by digestion of casein⁴ and a synthetic substrate⁵. Cells were treated with neuraminidase (Fig. 3, *Vibrio cholerae*, Behringwerke and washed three times.

Con A was purchased from Miles-Yeda; SBA and peanut agglutinin were purified by affinity chromatography as described elsewhere^{6,7}. The kinetics of agglutination⁸ were followed on a Cytograf Model 6301 electronic cell counter (Bio/Physics, Mahopac). Briefly, 10 ml of a 2% erythrocyte suspension ($\sim 10^8 \text{ cells ml}^{-1}$) in solutions of lectin in PBS at room temperature were agitated in scintillation vials on a New Brunswick gyrotary shaker at 75 r.p.m. Mixed agglutination was usually accomplished by mixing equal volumes of 2% suspensions of trypsinised and untrypsinised erythrocytes in PBS and adding the lectin. Samples ($50 \mu\text{l}$) were diluted 1:200 with PBS and counted immediately at non-agglutinated levels of about 40,000 particles per 0.1 ml. Replicate readings on duplicate dilutions for each time point were averaged.

The results obtained using $50 \mu\text{g}$ con A per ml of 2% erythrocyte suspension are shown in Fig. 1. Untrypsinised cells and cells treated with trypsin in the presence of soybean trypsin inhibitor (1:1 with trypsin) showed no agglutination. Cells treated with trypsin and then washed with or without trypsin inhibitor agglutinated within 15 min and reached equilibrium after 4–5 h. What is remarkable is that untrypsinised erythrocytes agglutinate when mixed with trypsinised cells as well as the trypsinised cells alone, even when the trypsinised cells were first washed with trypsin inhibitor. The agglutination was readily and specifically reversed by addition of 20 mM methyl α -D-mannopyranoside to the reaction mixture (arrow).

Fig. 1 The kinetics of erythrocyte agglutination using $50 \mu\text{g}$ con A per ml. ●, Untrypsinised erythrocytes; ■, trypsinised erythrocytes; ▲, mixed population of equal volumes of trypsinised and untrypsinised erythrocytes. After 17 h α -methyl-mannoside was added to a final concentration of 20 mM (arrow).



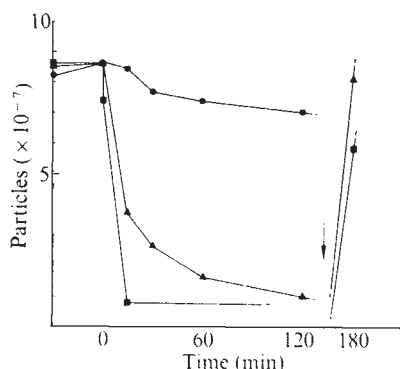


Fig. 2 The kinetics of erythrocyte agglutination using 30 μg SBA per ml. ●, Untrypsinised erythrocytes; ■, trypsinised erythrocytes; ▲, mixed population of equal volumes of trypsinised and untrypsinised erythrocytes. The mixed agglutination was started by dripping the trypsinised erythrocytes into the solution of untrypsinised erythrocytes and lectin over 10 min, to oblate the almost instantaneous agglutination of trypsinised erythrocytes with SBA. After 135 min, D-galactose was added to a final concentration of 20 mM (arrow).

The kinetics of agglutination using 30 μg SBA per ml of 2% erythrocytes was complicated by the tremendous speed with which trypsinised cells agglutinated with this lectin, even at concentrations as low as 5 μg SBA per ml (not shown). To allow time for the untrypsinised cells to agglutinate with the trypsinised cells, 5 ml of a 2% suspension of trypsinised cells was rapidly dripped into the rotating reaction vessel which contained 5 ml of a 2% untrypsinised erythrocyte suspension, and enough SBA to give a final concentration of 30 $\mu\text{g ml}^{-1}$ (Fig. 2). The results demonstrate that mixed agglutination of both untrypsinised and trypsinised erythrocytes was occurring. The reversibility of these cells on addition of 20 mM D-galactose was rapid and specific; 20 mM mannose or glucose was ineffective.

The mixed agglutination using con A and SBA was in striking contrast to that obtained with PNA. Erythrocyte T antigen has been shown to interact specifically with PNA⁷. Thus, neuraminidase-treated erythrocytes bind and agglutinate with PNA⁷. Untrypsinised and trypsinised erythrocytes do not bind and are not agglutinated by PNA

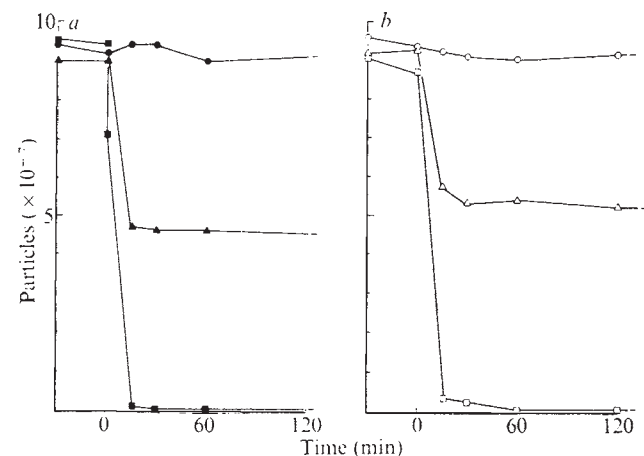


Fig. 3 The kinetics of erythrocyte agglutination using 30 μg PNA per ml. a, ●, Untreated erythrocytes; ■, neuraminidase-treated erythrocytes; ▲, mixed population of equal volumes of neuraminidase-treated and untreated erythrocytes. Neuraminidase treatment consisted of 60 min incubation in 20 units neuraminidase (*Vibrio cholerae*, Behringwerke) per ml 4% erythrocyte suspension in 0.9% NaCl containing 10^{-3} M CaCl_2 at 37°C; b, ○, trypsinised erythrocytes; □, neuraminidase-treated erythrocytes; ▲, mixed population of trypsinised erythrocytes and neuraminidase-treated erythrocytes. The results were as shown even when the neuraminidase-treated erythrocytes were dripped into a mixture of normal cells containing the lectin (as in Fig. 2).

even at very high concentration⁹. The results obtained using 30 μg PNA per ml in 2% erythrocytes are shown in Fig. 3a. Note that when neuraminidase-treated erythrocytes were mixed with untreated erythrocytes, the endpoint of the agglutination reaction fell halfway between the endpoints for neuraminidase-treated and untreated erythrocytes, indicating that no mixed agglutination had taken place. This indicates that trapping of non-agglutinating cells is not a factor in the con A and SBA results, and that trypsinisation does not automatically lead to enhanced agglutinability (Fig. 3b).

In contrast, when neuraminidase-treated erythrocytes are mixed with untreated erythrocytes in the presence of con A (50 $\mu\text{g ml}^{-1}$) or SBA (30 $\mu\text{g ml}^{-1}$), mixed agglutination occurred (Fig. 4), but to a slightly lesser extent than was seen with trypsinised and untrypsinised cells (Figs 1 and 2). These findings substantiate the observation that neuraminidase treatment of erythrocytes leads to an intermediate increase in lectin-mediated agglutinability³, as compared to trypsin treatment. We extend these observations to include the finding that the presumptive removal of surface charge from only one surface by neuraminidase permits substantial agglutination to occur with unaltered erythrocyte surfaces.

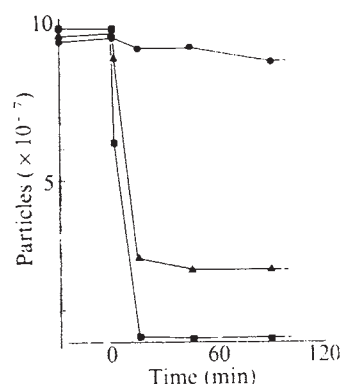


Fig. 4 The kinetics of erythrocyte agglutination using 30 μg per ml SBA. ●, Untreated erythrocytes; ■, neuraminidase-treated erythrocytes; ▲, mixed population of normal erythrocytes and neuraminidase-treated erythrocytes, where the latter cells were dripped into the normal cells containing the lectin (as in Fig. 2).

An explanation for the mixed agglutination is that residual trypsin, or neuraminidase, in the treated cell population acts on the untreated population. This seems highly unlikely, however, as (1) tryptic activity in solutions containing washed trypsinised cells was undetectable by two different assay methods (less than 0.5 $\mu\text{g ml}^{-1}$), (2) added soybean trypsin inhibitor with the trypsinised erythrocytes did not lessen the enhanced agglutination with untrypsinised cells, and (3) washed neuraminidase-treated erythrocytes showed no mixed agglutination with untreated cells in the presence of peanut lectin.

Two findings are worthy of mention. First, the mixed agglutination with two lectins is enhanced by proteolytic or neuraminidase treatment. This would suggest that cell alterations in addition to the simple removal of glycopeptides by trypsin or the simple removal of surface charge by neuraminidase from all cells must be involved¹⁻³. Furthermore, a specific binding by lectin to treated and untreated cells seems necessary, as PNA does not show mixed cell agglutination to cells to which it does not bind, even if that cell is trypsinised (Fig. 3b).

One feasible explanation of our results is that neuraminidase or trypsin treatment leads to an increase in erythrocyte deformability and thereby increases the total area of membrane contact with untreated cells. Our preliminary experiments indicate that trypsin treatment increases the total erythrocyte deformability in PBS when measured by the ability of erythrocytes to penetrate poly-

carbonate filters¹⁰ of 5 μm pore size using gravity flow. Using micropipette elastimetry, Weiss *et al.*¹¹ have shown clearly that the membrane of nucleated cells is more easily deformed by a local perturbation following tryptic treatment. Neuraminidase also apparently increases the local deformability of erythrocytes¹². Whether erythrocytes show greater local membrane flexibility following trypsinisation remains unclear. In any event, the observations reported here of enhanced mixed cell agglutinations, both kinetically and at equilibrium, suggest that explanations of enhanced cell agglutination based primarily on the removal of surface polypeptides or loss of surface charge¹⁻³, or simple changes in affinity constants¹³ of the total cell population, must be reconsidered.

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Polarity of actin at the leading edge of cultured cells

VERTEBRATE non-muscle cells are known to contain considerable amounts of actin and myosin¹⁻³, but the mechanisms underlying their motility have yet to be elucidated. Various theories have been proposed^{1,4-7} to explain the well known phenomena of cell migration, membrane ruffling and unidirectional growth processes, different emphasis being placed on the involvement of membrane components and contractile filament assemblies. These hypotheses have been limited, however, by the lack of information about the detailed structural organisation of the motile regions of the cytoplasm. As one approach to this problem we have developed procedures for the direct observation of the filamentous components of the lamella regions of cultured cells in the electron microscope^{8,9}. The method, similar to that adopted by Brown *et al.*¹⁰, consists of the negative-staining of cells grown directly on electron microscope grids after removal of the cell membranes with Triton X-100. By this means, we demonstrate here a single polarity of actin at the leading edge of cultured cells, and consider its implications with regard to cell motility.

The filamentous organisation within advancing regions and leading lamellae were investigated in different cell lines: BALB 3T3 cells, human skin fibroblasts and neuroblastoma cells, primary attention in the latter cells being paid to the organisation of the neuronal growth cones. Two clearly distinct specialisations of actin could be recognised in these motile regions (Fig. 1), a planar diagonal meshwork of actin filaments between about 1 μm and 3 μm in width, from which individual filaments were seen to project, and actin filament bundles about 0.1 μm in diameter which originated and projected radially from the actin meshwork. These latter bundles, which evidently corresponded to the microspikes or filopodia described in earlier investigations¹¹ were frequently seen, under identical

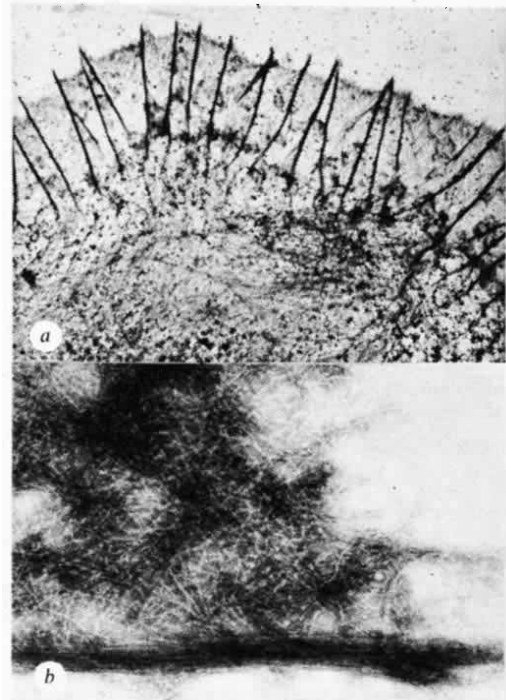
conditions, in the growth cones of neuroblastoma cells and in skin fibroblasts, but were less prominent in 3T3 cells, in which the leading lamellae sometimes showed only the filament meshwork and no filament bundles. While former studies⁷ had shown the presence of a meshwork of actin in the motile outgrowing regions of cultured cells, the filaments in these areas in normal thin sections seemed to be randomly arranged. The present observations show that the filaments of this meshwork form a planar criss-cross network, with individual filaments arranged obliquely with respect to the direction of cell advancement.

In some cells, the advancing edge was seen to be folded back on itself, and such regions were interpreted as sites of membrane ruffling. The organisation of thin filaments in these regions was apparently the same as described for the leading edge. In the conditions used for Triton extraction and fixation, which were previously found to preserve the myosin filaments of smooth muscle¹², no myosin-like aggregates were observed in the cultured cells, either in the leading edge or elsewhere in the cytoplasm.

By the addition of myosin subfragment-1 to the cells following Triton extraction, it was found that not only did this subfragment bind, as expected, to filaments in the meshwork and the microspikes, identifying them as actin, but that the filaments in both these locations possessed the same polarity with respect to the cell body (Fig. 2). In all cases the arrowheads were directed towards the cell body, that is, away from the direction of advancement of the leading edge. The polarity of the arrowhead complex was most readily visualised at the tips of the microspikes, where the filaments became fewer in number or more loosely packed, and on the filaments projecting from the anterior edge of the diagonal meshwork.

Studies on the microvilli of intestinal epithelial cells¹³ have revealed a unipolar organisation of actin, with the arrowhead complexes formed from binding with myosin subfragment-1 also directed inwards towards the cell body.

Fig. 1 *a*, Leading lamella of human skin fibroblast after Triton extraction and negative staining, showing the leading edge as a broad meshwork with radiating microspikes. *b*, Detailed organisation of the leading edge. Co-linear bundle of actin filaments (lower part of micrograph) arises from the diagonal meshwork of thin filaments. Conditions: 0.1% Triton X-100 for 60 s at room temperature followed by a Triton-free rinse, fixation in glutaraldehyde and negative staining in the cold with uranyl acetate⁷. Magnifications: *a*, $\times 3,900$; *b*, $\times 66,000$.



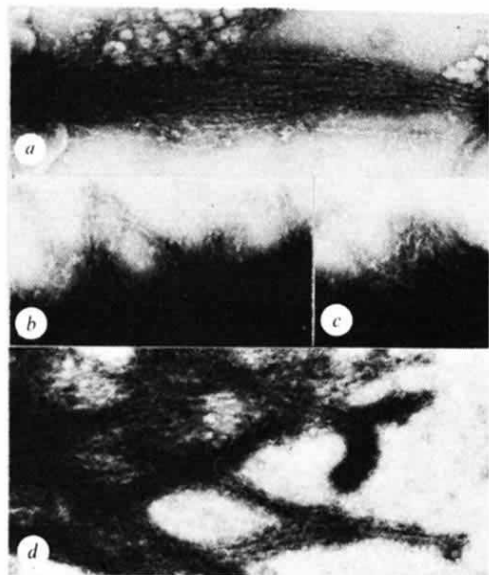


Fig. 2 Acto-S-1 complexes formed between myosin subfragment-1 and the components of the leading edge of human skin fibroblasts. *a*, The tip of a radiating microspike; *b*, *c*, the anterior edge of the thin filament meshwork; *d*, acto-S-1 complex between myosin subfragment-1 and the anterior region of a neuronal growth cone of a cultured neuroblastoma cell. The arrowhead complex is directed, in all cases, away from the direction of cell movement. Conditions^{7,8}: cells extracted with Triton X-100 were washed in Triton-free medium, incubated for 30–60 s in 1 mg per ml smooth muscle myosin subfragment-1, washed again, fixed in glutaraldehyde and negatively stained. Magnifications: *a*, $\times 67,000$; *b*, *c*, $\times 74,000$; *d*, $\times 60,000$.

Also, more recently, the microspikes of eggs¹⁴ and coelomocytes¹⁵ of the sea urchin have been shown to exhibit the same polarity. We have now been able to demonstrate that a unipolar organisation of actin is a characteristic feature of the advancing lamellae of cultured cells, not only in the microspikes but also in the interconnecting web or meshwork of actin filaments. It is apparent also that the latter meshwork constitutes the primary requirement for motile activity.

From these observations, we propose a scheme for the movement of cultured cells over the substratum similar to that put forward by Huxley¹, but with some significant modifications. Briefly, we suggest that advancement of the leading edge involves a shearing of substrate-associated actin with monomeric myosin or unipolar aggregates of myosin coupled with actin polymerisation at the anterior edge. The shearing process would bring monomeric actin and membrane precursor components^{3,6,16} to the tip of the advancing edge for the forward polymerisation of actin filaments and for the formation of new membrane. Thus, rather than polymerising inwards from the cell membrane¹, actin would polymerise in a forward direction at the anterior edge of the meshwork and at the tips of the microspikes. Such a direction of polymerisation, opposite to that of the actin arrowhead complex, would be consistent with that observed for muscle actin *in vitro*^{17,18}. Membrane ruffling could be explained by the same shearing mechanism operating in the absence of binding of the terminal meshwork to the substrate, together with a subsequent depolymerisation of actin as the ruffles dissolve into the cell body. In echinoderm sperm, actin polymerisation alone has been shown to be the force-producing mechanism underlying the acrosomal reaction³. Further work will clearly be necessary to establish the relative contributions of actin polymerisation and actomyosin shearing to the motility of vertebrate non-muscle cells.

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Transfer of demyelination by intraneural injection of experimental allergic neuritis serum

EXPERIMENTAL allergic neuritis (EAN), an autoimmune demyelinating disease of peripheral nerve, and a model of human inflammatory polyneuritis (Guillain-Barré syndrome), can be produced by immunisation with peripheral nerve tissue in complete Freund's adjuvant (CFA)^{1,2}. EAN, like its central nervous system counterpart, experimental allergic encephalomyelitis (EAE), has been considered as the result of cell-mediated immunity^{3,4}, which can be transferred with lymphoid cells^{4,5}. Sera from animals with EAN or EAE induced by whole nervous tissue antigen are capable of demyelinating myelinated organotypic cultures of peripheral or central nervous system origin, respectively^{6,7}. The demyelinating factor in EAE sera is immunoglobulin^{8,9}. However, little evidence has been presented to suggest that the demyelinating antibodies which can be demonstrated *in vitro* are involved in the pathogenesis of these diseases *in vivo*^{4,10–12}. EAN and EAE have not been transferred passively with serum from affected to recipient animals by systemic^{4,11} or intravitreal injection¹². We report here the first successful transfer of demyelination with EAN serum. Antiserum was injected directly into nerve parenchyma of recipient animals to circumvent the blood-nerve barrier. The demyelination was associated with macrophages but not with lymphocytic infiltration. The ability of sera to transfer demyelination correlated with their capacity to demyelinate cultured dorsal root ganglia. Organ specificity and complement dependency strongly suggested that the reaction was antibody-dependent and complement-mediated.

EAN sera were obtained at various times post-immunisation (Table 1) from seven rabbits sensitised by bovine or human femoral nerve in CFA. Control sera were collected from 10 rabbits similarly inoculated with bovine liver or kidney, or with serum albumin in CFA or CFA alone, and seven pre-immunisation (EAN) rabbits. One group of recipient animals, 46 rats and three rabbits, received injections of 40 or 16 μ l of EAN serum into one sciatic nerve, and the same volume of control

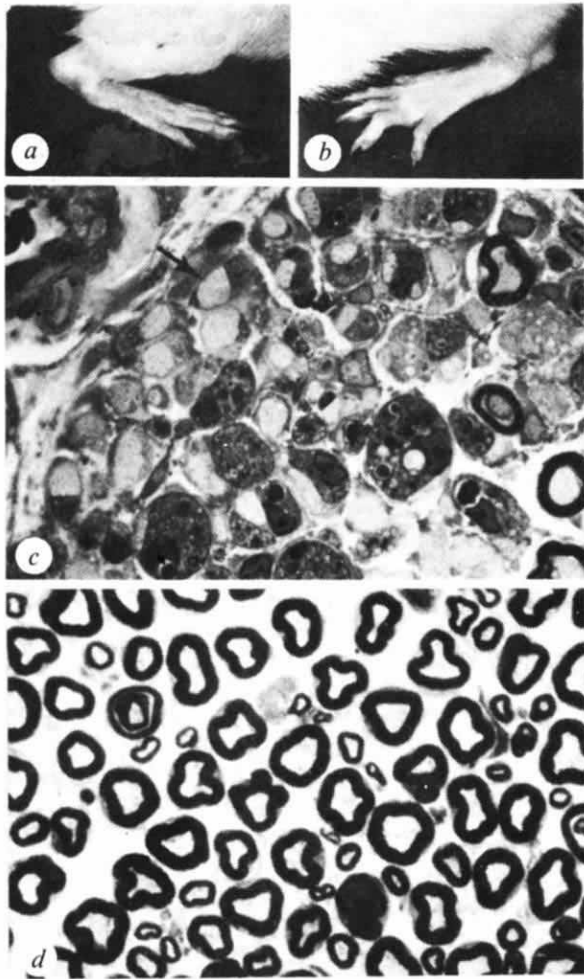


Fig. 1a, Paralysis of foot and toe of rat 2 d after injection of 40 µl EAN-rabbit serum (BN3-III in Table 1) into the ipsilateral sciatic nerve; b, contralateral hind limb remains normal after intrasciatic nerve injection of serum from a rabbit inoculated with CFA alone; c, 1-µm cross section of an Araldite-embedded rat sciatic nerve 5 d after injection with EAN serum, stained with 1% toluidine blue. Demyelinated axons (arrow) are invested in debris-laden macrophages (×1,000). d, Contralateral sciatic nerve injected with control serum shows nerve fibres slightly dispersed by endoneurial oedema. No demyelination or cell infiltration are present (×900).

serum into the contralateral nerve. Serum was injected under the perineurium using a 50-µl syringe with a 30-gauge disposable needle.

When EAN sera obtained later than 14 d post-immunisation were used, most recipient rats and rabbits developed foot weakness of varying degree and decreased response to pinprick stimuli on the injection side (Fig. 1a). Clinical abnormalities occurred several hours following injection, became maximal within 2 d, and gradually subsided after the third day. Paresis and hypoesthesia were never seen on the side injected with control serum (Fig. 1b). Recipient sciatic nerves were fixed with glutaraldehyde at multiple intervals between 20 min and 8 d after injection, excised, postfixed with OsO_4 , and studied by light and electron microscopy.

Nerves injected with 12 out of 13 EAN sera taken later than 14 d post-immunisation showed a demyelinating lesion around the site of injection (Table 1). The earliest demyelination, splitting and vacuolation of myelin lamellae, was seen 1 h after antiserum administration, and vesiculation of myelin appeared within 5 h. Polymorphonuclear cells and severe oedema were found in the endoneurium within several hours, and by 15 h invasion of monocytes and macrophages became prominent. Macrophages attached to, surrounded and phagocytosed myelin (Fig. 1c, 2), often insinuating their processes

between myelin lamellae. The findings were indistinguishable from the demyelinating patterns observed in EAN animals¹³⁻¹⁵. Control serum-injected nerves showed mild endoneurial oedema but no demyelination (Fig. 1d). Mechanically distorted myelin sheaths and wallerian degeneration were seen only along the needle track.

To compare the *in vitro* demyelinating activity of 32 EAN or control sera with their ability to transfer *in vivo* demyelination, 40% test sera were applied to well-myelinated mouse dorsal root ganglion cultures¹⁶ (Table 1). *In vitro* demyelinating activity of each serum correlated well with its ability to transfer *in vivo* demyelination.

To evaluate specificity of the *in vivo* demyelinating activity, three active sera were incubated for 1 h at 37 °C and overnight at 4 °C with 10% (v/v) lyophilised purified bovine peripheral nerve myelin, minced fresh bovine femoral nerve, liver, or kidney (Table 2). Demyelinating activity in the recipient rat nerves was absorbed by peripheral nerve myelin or crude peripheral nerve, but not by liver or kidney.

To study complement dependency of the *in vivo* demyelinating reaction, the same sera were heated at 56 °C for 30 min and injected into nerves of recipient rats which had been deplemented by injection of cobra venom factor¹⁷ (Table 2). Heated sera did not produce demyelinating lesions unless unheated guinea pig serum was added to the inoculum as a source of complement. We found that after freezing of EAN sera at -20 °C for 2 weeks, demyelinating activity was still demonstrable. This contrasts with the reported instability of EAE-

Table 1 Comparison of demyelinating activities of EAN and control sera assessed by intraneural injection and dorsal root ganglion (DRG) culture application

Serum*	Demyelinating activity†	
	In rat sciatic nerve	In mouse DRG § culture
Control (n = 17)	-	-
BN2-I	-	-
BN3-I	-	-
BN2-II	+	+
BN3-II	++	+++
HN1-II	+	NT
BN1-III	+	+
BN2-III	++	++
BN3-III	+++	+++
BN4-III	+	+
BN5-III	++	++
HN1-III	+	++
HN2-III	+	++
BN3-IV	++	++
BN2-V	+	NT
HN1-V	-	NT

*Antisera were produced in male New Zealand albino rabbits weighing 2.3-2.7 kg. The inoculum to produce EAN contained 0.2 g bovine (BN) or human nerve (HN) homogenate, 0.2 ml saline, 0.4 ml CFA (Difco) containing 0.4 mg heat-killed H37Ra *Mycobacterium tuberculosis*. All seven EAN rabbits developed clinical manifestations of EAN after 15-31 d. EAN sera were obtained 7 d (I) and 14 d (II) after sensitisation, and within 1 d (III) and 7 (IV) and 14 (V) d after the clinical onset of disease. Control sera were obtained from seven preimmunisation (EAN) rabbits and 10 rabbits injected with non-neural materials, bled around 20 d postinoculation. All sera were stored at -70 °C and thawed once just before testing.

†*In vivo* and *in vitro* demyelinating activity were determined independently by different observers in a coded fashion.

‡To determine *in vivo* activity, 26 male Wistar rats weighing 200-250 g received injections of 16 µl of either EAN or control serum and 4 µl of guinea pig serum as a source of complement, and were killed after 48 h. Activity was expressed by an estimate of the ratio of demyelinated area to whole fascicular area of a cross section at the site injected, as follows: +++ , >30%; ++ , 10-30%; + , <10%; -, only a few or no nerve fibres with myelin destruction.

§To determine *in vitro* activity of each test serum, 4-7 cultures¹⁶ were selected and observed with a light microscope for 3 d following exposure to 40% test serum, 10% guinea pig serum, and 50% nutrient medium. Demyelinating activity was evaluated as follows: +++ , severe (total demyelination after 24 h); ++ , moderate (extensive demyelination after 3 d); + , mild (minimal demyelination after 3 d); -, no demyelination (after 3 d).

Table 2 Demyelinating activity of EAN sera after absorption or heat treatment (56 °C for 30 min)

EAN serum	Testing method*	Myelin	Nerve	Demyelinating activity*			
				Absorbed serum†	Liver	Kidney	Heated serum‡
						‡Heated GPS	‡Unheated GPS
BN2-III	In nerve	-	-	+	+	-	+
	In culture	-	-	+	+	-	++
BN3-III	In nerve	-	-	++	+	-	++
	In culture	-	-	++	++	-	++
BN5-III	In nerve	-	NT	+	+	-	++
	In culture	-	NT	+	+	-	++

*Activity was tested as described in Table 1, using identical EAN and guinea pig serum (GPS) concentrations.

†For absorption studies unheated GPS was added to the supernatant of centrifuged preincubated serum for both *in vivo* and *in vitro* tests.

‡For complement-dependency studies heated or unheated GPS was added to heated EAN serum for *in vivo* and *in vitro* tests. Recipient rats were complement-depleted by intraperitoneal injection of cobra venom factor (Cordis), 250 units per kg body weight, in four divided doses over the 24 h before injection.

lymph node cell supernatant activity which can produce focal perivascular mononuclear cell infiltrates in recipient brain and spinal cord¹⁸.

The fact that serum activity was produced only by sensitisation with nerve tissue and was absorbed only by myelin or nerve indicates the organ specificity of this serum transfer. Organ specificity and complement dependency suggest the involvement of immunoglobulin in both the *in vivo* reaction and the analogous situation in cultured peripheral nerve. The earliest demyelination may result from an antibody-dependent complement-mediated reaction, with secondary attraction of invading cells which augment the process. We successfully transferred the most conspicuous feature of demyelinating disorders, demyelinating lesions accompanied by infiltration of macrophages, monocytes and polymorphonuclear cells. However, lymphocytic infiltrations were absent; therefore, the lesions should not be interpreted as a sign of replication of the complete EAN lesion or of a delayed hypersensitivity reaction, in which early perivenular lymphocytic infiltration is one of the characteristic components^{2,19}. In contrast, the only report of passive transfer of EAE with antiserum describes replication of a delayed hypersensitivity reaction 3 d after intraventricular injection of serum²⁰.

Fig. 2 Cross section of rat peripheral nerve fibre 14 h after intraneural injection of EAN serum. Macrophage (M) or possibly retracted Schwann cell cytoplasm (lower M) within the Schwann cell basement membrane (double arrows) surrounds myelin sheath undergoing splitting and vesicular disruption (arrow).

Stained with uranyl acetate and lead citrate. ($\times 15,000$).



Altered permeability properties of neural capillaries in EAN have been reported^{3,13-15,21}, so that serum-mediated demyelination may take place in actively sensitised EAN animals following disturbance of the blood-nerve barrier. Alternatively, antibodies produced locally by infiltrating lymphocytes and plasma cells could contribute to antibody-dependent complement-mediated damage. The presence of mononuclear phagocytes in the demyelinating lesion of actively sensitised animals¹³⁻¹⁵ is thus compatible with an antiserum-mediated demyelinating process. It is possible that demyelinating antibodies have an important role in the pathogenesis of demyelination in EAN, as well as the well-recognised and probably primary role of cell-mediated immunity.

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α -Bungarotoxin enhances transmitter 'released' at the neuromuscular junction

α -BUNGAROTOXIN (α -BTX), a neurotoxin obtained from the venom of the snake *Bungarus multicinctus*, blocks neuromuscular transmission by combining irreversibly with the acetylcholine (ACh) receptors located in the postsynaptic membrane¹⁻⁴. The results reported here show that α -BTX

also causes an increase in the amount of ACh collected after nerve stimulation.

Rat nerve hemidiaphragms were kept at room temperature (18–22 °C) in Ringer solution (mM: NaCl 116; KCl 4.5; NaCO₃ 23; NaH₂PO₄ 1; CaCl₂ 2; MgCl₂ 1; glucose 11) bubbled with 95% O₂+5% CO₂. In early experiments the solution contained 20 μ M diisopropylfluorophosphate (DFP) to inhibit cholinesterase irreversibly. However, the continued presence of DFP was found to produce some undesirable effects. For instance, DFP reduced the amplitude of miniature endplate potentials, through a postsynaptic action, and also produced a presynaptic block in the propagation of impulses to some endplates. These effects were reversible, and in later experiments the DFP was washed out after the cholinesterase was fully inhibited, before collecting the ACh released from the tissue into the 5 ml bath. The collection periods were 20 or 30 min during rest and 15 min during nerve stimulation. ACh in the fluid was measured, as described previously^{5,6}, by pyrolysis mass fragmentography using deuterated ACh as internal standard.

As illustrated in Fig. 1, the hemidiaphragm spontaneously released about 0.5 pmol ACh min⁻¹, and when the nerve was stimulated at 3 Hz the amount of ACh released into the fluid increased about threefold. Addition of α -BTX to a concentration of 5 μ g ml⁻¹ rapidly blocked the ACh receptors, and within 20 min endplate potentials were undetectable in superficial muscle fibres. At this time, due to the slower access of α -BTX to deeper endplates³, some fibres still contracted in response to nerve stimulation but all neuromuscular transmission was blocked 40–80 min after introducing the toxin. When transmission had been blocked completely, the unbound α -BTX was removed by thorough washing before collecting the ACh. α -BTX treatment did not significantly alter the resting release of ACh (Table 1). In contrast, the amount of ACh collected after

Fig. 1 Effects of α -BTX on the amount of ACh released from rat hemidiaphragms into the incubating medium. Before collection of ACh, the muscles were incubated for 80 min in the presence (dotted line) or absence (continuous line) of 5 μ g ml⁻¹ α -BTX. During this preincubation period the Ringer also contained 20 μ M DFP. The preincubation period was terminated by extensive washings of the muscle with Ringer. ACh was assayed by mass fragmentography^{5,6}. ACh, which was released spontaneously, was collected in two 0.5-h periods; subsequently, during four collection periods of 15 min, the phrenic nerve was stimulated supramaximally with 0.05-ms pulses at 3 s⁻¹, indicated by the bar.

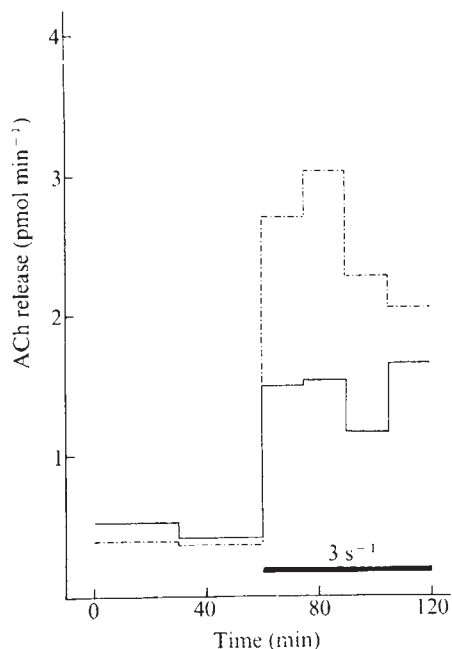


Table 1 Influence of α -BTX on spontaneous release of ACh and on the release evoked by electrical and K⁺ stimulation in rat hemidiaphragm

	(n)	Spontaneous or evoked release of ACh		
		a Untreated control (pmol min ⁻¹)	b Treated with 5 μ g ml ⁻¹ α -BTX (pmol min ⁻¹)	c b/a
Ringer	14	0.5 $\pm$ 0.04	0.4 $\pm$ 0.04	
Ringer, 3 s ⁻¹ stimulation	8	1.3 $\pm$ 0.13	2.1 $\pm$ 0.12†	1.70 $\pm$.20
Ringer, 1.6 μ M TTX	12	0.4 $\pm$ 0.05	0.5 $\pm$ 0.07	
Ringer*, 1.6 μ M TTX + 50 mM KCl	14	1.5 $\pm$ 0.14	2.8 $\pm$ 0.33†	1.90 $\pm$.19

Rat hemidiaphragms were incubated as described in Fig. 1. Values for ACh release evoked by electrical pulses or by K⁺ were obtained by subtracting from the total release the spontaneous release measured in the preceding periods. Values are mean $\pm$ s.e.m. The right column (c) gives the mean $\pm$ s.e.m. of the ratio between ACh collected in the α -BTX-treated diaphragm and its control.

*Ringer with reduced NaCl.

†Values significantly different from controls ($P < 0.005$).

nerve stimulation was significantly greater than that collected from the opposite hemidiaphragm, used as control (Fig. 1). Left hemidiaphragms tended to release more ACh than their right counterparts. Although this difference was not statistically significant, controls were taken alternately from right and left to avoid the possibility of errors introduced by asymmetric behaviour of the muscles. This was carried out for the experiments described in Table 1, which shows that α -BTX approximately doubled the amount of ACh collected after nerve stimulation.

Mammalian muscles treated with an anticholinesterase show spontaneous contractions due to the generation of impulses in some motor nerve terminals^{7,8}. Moreover, in these conditions a single stimulus to the nerve is often accompanied by repetitive firing of the terminals, and this could lead to fatigue and a reduction of the evoked release of ACh. As α -BTX was found to abolish spontaneous twitching and repetitive firing, it was conceivable that the increased output of ACh after toxin treatment was simply due to suppression of spontaneous activity and concomitant recovery of the release process from a depressed state. To check this, further experiments were carried out in the presence of tetrodotoxin (TTX), which is known to block all nerve and muscle impulse activity without interfering with the release of ACh from motor nerve terminals⁹. After nerve impulses were abolished by TTX, we depolarised the terminals directly with 50 mM KCl, in order to evoke transmitter release. As shown in Table 1, the spontaneous release of ACh was not decreased by TTX treatment, indicating that spontaneous impulses do not contribute appreciably to the amount of ACh discharged into the incubating medium. Furthermore, Fig. 2 and Table 1 show that the K⁺-induced release of ACh, like that induced by nerve impulses, was approximately doubled by treatment with α -BTX.

Our results differ from those of previous workers^{10–12}, who did not detect a change in the amount of ACh released from skeletal muscle after treatment with curare, a drug which, like α -BTX, blocks ACh receptors. It may be that α -BTX and curare have different actions on ACh release, but in preliminary experiments we have found that when diaphragms were incubated in a high (10⁻⁴ M) concentration of curare, the evoked (but not the spontaneous) release of ACh was doubled. On the other hand, our results resemble those found in other systems in which receptor blockers enhance the evoked release of transmitters. For instance, phenoxybenzamine or phentolamine, blockers of the adrenoceptor, stimulate the release of

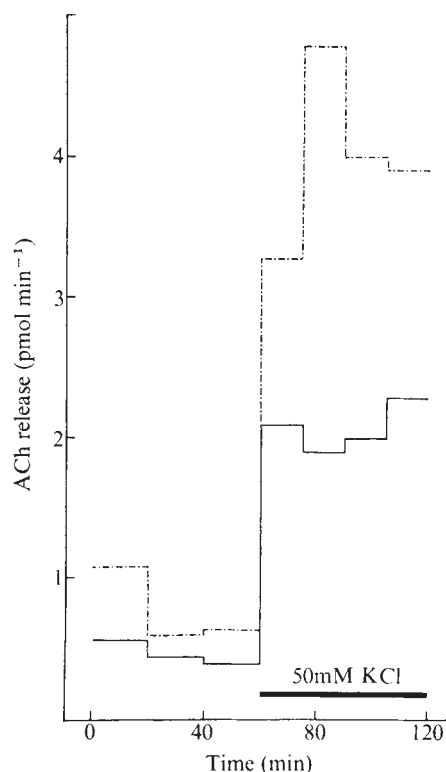


Fig. 2 Effect of α -BTX (dotted line) on release of ACh in the presence of $1.6 \mu\text{M}$ TTX. Control, continuous line. The spontaneously released ACh was collected in three periods of 20 min. Subsequently, during four periods of 15 min, the Ringer contained 50 mM KCl (indicated by the bar) and 71 mM NaCl, to depolarise motor nerve terminals.

noradrenaline in adrenergic systems^{13–15}; and atropine, a blocker of muscarinic acetylcholine receptors, enhances the release of ACh from brain cortex^{16–19}.

The mechanism whereby α -BTX increases the amount of ACh collected during nerve stimulation is not yet clear. One possibility is that the amount of ACh discharged from the nerve terminals is unaltered by α -BTX, but that normally some of the ACh is taken up by muscle fibres²⁰. Such uptake might be prevented by blocking the ACh receptors located in the muscle fibre membrane, and this, together with accelerated diffusion of ACh out of the synaptic cleft (which occurs after blockage of the ACh receptor²¹), would increase the amount of ACh escaping into the surrounding fluid.

Another possibility is that the process of transmitter release may be inhibited by a pre- or postsynaptic effect of the ACh which is continuously leaking from nerve terminals²², or by the ACh released by nerve impulses. If this inhibition were removed by α -BTX, the number of transmitter packages released by nerve impulses would be increased. For instance, it has been reported that stimulation of postsynaptic ACh receptors leads to a release of ATP^{23–25}, and it is possible that this ATP inhibits further ACh release^{25, 26}. Alternatively, it could be that the terminals possess ACh receptors which when activated exert an inhibitory influence on ACh release. A feedback system by either of these mechanisms could have interesting implications on the functioning of neuromuscular synapses.

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Electrical responses by guinea pig megakaryocytes

THE striking similarities between blood platelets and muscle cells elucidated in recent years (for example, contractile properties, a full complement of contractile proteins, a dual canalicular system resembling the sarcoplasmic reticulum of muscle, and an active intracellular calcium pump; for review see ref. 1) suggests that the platelet might also manifest electrical activity resembling that seen in muscle. Unfortunately, the small size of the platelet ($2\text{--}4 \mu\text{m}$ in diameter) has precluded study of transmembrane electrical potentials using available techniques. However, as circulating platelets represent cytoplasmic fragments of the much larger megakaryocytes, we reasoned that the megakaryocyte itself might well possess interesting electrophysiological properties. The studies described here represent our initial characterisation of membrane responses in the megakaryocyte.

Suspensions of guinea pig marrow cells were obtained by modifications of the method of Levine and Fedorko² (see Fig. 1 for details). Electron microscopic evaluation of the suspensions confirmed the presence of well preserved megakaryocytes. A small aliquot of the suspension was added to the bathing solution in a plastic culture dish. Megakaryocytes, which were readily identified on the basis of their large size and characteristic multilobed nuclei, were penetrated with a single microelectrode by increasing the negative capacitance until the amplifier oscillated once the electrode had made initial contact with the cell membrane. Following an abrupt penetration, transmembrane potentials could usually be monitored for at least several minutes, and occasionally for 20 min or more. Only cells showing stable membrane potentials and input resistances were included in the study.

The resting potentials varied in a consistent fashion with changes in extracellular calcium (Fig. 1): the values were low with calcium concentrations in the physiological range (2.4 mM), but were more negative with higher calcium concentrations. By contrast, increased calcium produced no progressive increase or decrease in input resistance (data

not shown). This suggests that the increased resting potentials resulted from a combination of factors rather than simply a reduction in membrane damage during penetration or alteration in the conductance to a single ion.

As has been observed in several other mammalian cells of mesenchymal origin^{3,4}, many megakaryocytes tested (41/79 in 9 experiments) displayed long lasting hyperpolarisations (2–10 s) either spontaneously or in response to very large, relatively brief negative current pulses (Fig. 2). These hyperpolarising responses were variable in amplitude (5–40 mV) and were accompanied by marked decreases in membrane resistance. They occurred primarily in cells with low resting potentials ($\bar{x} = -13.5$ mV) and consequently were more common at low calcium concentrations. In some cases (such as that shown in Fig. 2b), the cell was initially

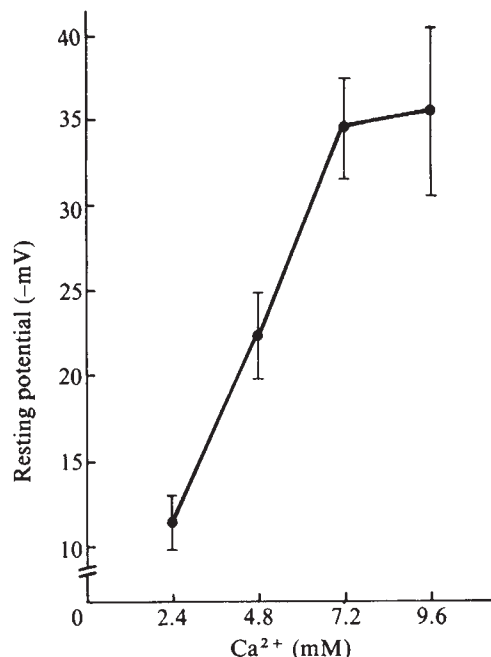


Fig. 1 Resting membrane potentials in megakaryocytes exposed to varying extracellular Ca^{2+} concentrations. Megakaryocytes were obtained as follows. Femurs and tibia were removed from 200–400 g guinea pigs anaesthetised with nembutal. Marrow from these bones was taken into CATCH suspending solution, which significantly enhances the yield of intact megakaryocytes². This medium consisted of calcium- and magnesium-free Hanks' balanced salt solution (GIBCO) to which was added sodium citrate (0.38% w/v), adenosine (1 mM), theophylline (2 mM) and bovine serum albumin (1% w/v; Fraction V, Sigma); pH was adjusted to 7.4. The marrow was minced, dispersed by repetitive pipetting, and passed through a 100 mesh stainless steel screen to remove large pieces of remaining tissue. The filtrate was subsequently washed twice by centrifuging for 10 min at 250g and resuspending in CATCH medium. After the final spin, the pellet was resuspended in approximately 3–5 ml CATCH, so that the addition of a 0.02-ml aliquot to 2.5 ml bathing solution in the recording chamber (35-mm polystyrene culture dish, Falcon) resulted in a 125-fold dilution of citrate, adenosine and theophylline, while still providing adequate numbers of megakaryocytes for study (approximately 5–20 well isolated megakaryocytes per dish). The bathing solution consisted of calcium- and magnesium-free Hanks' to which was added CaCl_2 (2.4–12 mM final concentration) and bovine serum albumin (1% w/v), and which was maintained at pH 7.2–7.6. Microelectrodes were filled with 3M KCl plus 0.1 M Kacetate by the glass-fibre technique⁸ and had a resistance of 50–80 M Ω . A bridge circuit built into the amplifier (M4A; WPI, Hamden) permitted the passage of current pulses through the same electrode used for recording. Voltage changes due to electrode resistance were balanced out by conventional methods and the currents were generally within the linear range of the electrodes. None of the electrical responses recorded intracellularly could be reproduced with the electrodes outside the cells, even with very large current pulses (greater than in Fig. 4). Mean resting potential values $\pm$ s.e.m. (inside negative) are plotted against Ca^{2+} concentrations, which were varied by adding CaCl_2 solution as described above.

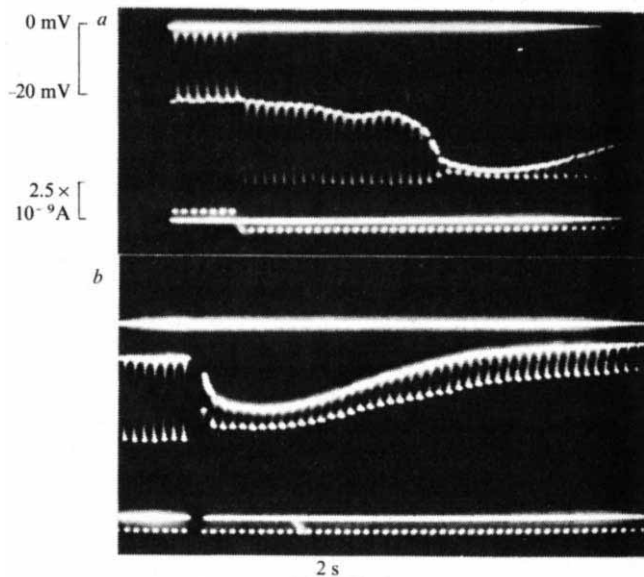
'silent'; but after a few hyperpolarising responses were elicited by current pulses, the cell began to produce spontaneous hyperpolarisations at irregular intervals.

The amplitudes of the hyperpolarisations in a few cells were altered by adjusting the membrane potential to different levels with d.c. current. With increasingly more hyperpolarised membrane potentials, the induced hyperpolarising responses decreased in amplitude; moreover, when the membrane was sufficiently hyperpolarised, the sign of the response reversed, indicating the presence of a reversal potential. The level of the apparent reversal potential was consistent with a predominant increase in potassium conductance such as has been implicated in similar hyperpolarising responses in fibroblasts³ and macrophages⁴.

Stimulation of some megakaryocytes with depolarising pulses of current, by contrast, produced 'active' membrane depolarisations. These responses were generally seen in cells exposed to higher calcium concentrations (≥ 4.8 mM) and thus having more negative resting potentials ($\bar{x} = -39.5$ mV). In some cells (25/79 in 9 experiments), stimulation produced responses somewhat resembling action potentials of nerve (for example, Fig. 3e), often including a depolarising peak overshoot and a hyperpolarising undershoot following the stimulus. In contrast to the action potentials of nerve or skeletal muscle, however, membrane depolarisations outlasting the stimulus were not observed. Furthermore, the responses usually tended to be graded (Fig. 4c) rather than strictly all-or-nothing, although in occasional cells a fairly constant response was obtained after threshold was surpassed (for example, Fig. 3c and d).

A number of cells (22/79) required depolarisation beyond 0 mV before a response was elicited (Fig. 4a–c). Typically in such cases the peak depolarisation was maintained and no repolarisation phase was observed during the current pulse, nor was a hyperpolarising undershoot seen following

Fig. 2 Spontaneous (a) and induced (b) hyperpolarising responses. A megakaryocyte was impaled with a single microelectrode through which brief hyperpolarising or depolarising current pulses were passed at 4 per s to monitor membrane resistance. The cell initially had a resting potential of -10 mV (b) and an input resistance (voltage/current) of 26.2 M Ω . A large hyperpolarising current pulse (offscale) induced a prolonged hyperpolarisation accompanied by a marked decrease in input resistance (down to 5.2 M Ω). The membrane potential returned slowly to the resting level followed by a restoration of the input resistance (not shown). With time the resting potential gradually became more negative (a) and the cell began to produce spontaneous hyperpolarisations and decreases in input resistance. The medium contained 2.4 mM Ca^{2+} . Calibrations apply to both a and b.



the stimulus. Occasionally a cell was found which appeared to exhibit this type of waveform as a second component of a more complex response (Fig. 4e). In this event the response to lower levels of stimulating current (Fig. 4d) did not in itself seem capable of triggering the second component; rather, further depolarisation as provided by the stimulating electrode, seemed to be necessary (Fig. 4e). Whether or not these dual responses actually represent contributions by separate current carriers is not known.

Our findings represent the first report, to our knowledge, that haematopoietic cells can give 'active' depolarising responses to electrical stimuli. That a precursor cell to the blood platelet should be capable of responses resembling those encountered in 'excitable' cells is in itself intriguing

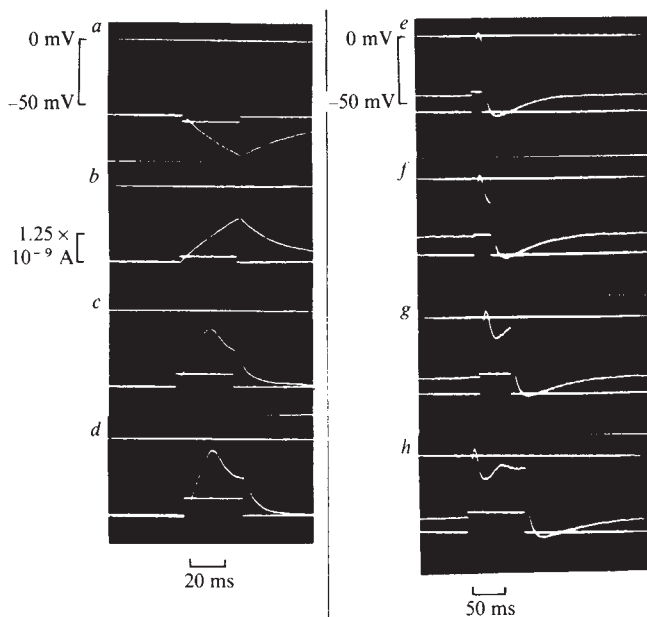


Fig. 3 Depolarising 'active' responses induced by small depolarising stimuli. *a* and *b* passive hyperpolarisation and depolarisation, respectively; *c* and *d*, suprathreshold responses to two different stimulus intensities. Slight inflections on the rising phases suggest these are regenerative responses. *e-h*, Responses of the same cell shown in (*a-d*) to stimuli of constant intensity, but gradually increasing duration. In the time interval between the two series, the resting potential depolarised slightly, revealing a pronounced undershoot following each stimulus. In *g* and *h* there is a definite repolarisation and partial recovery following the overshooting peak response and in *h*, even a slight oscillation. The medium contained 9.6 mM Ca^{2+} . Current calibration applies to all traces. Note differences in time calibration.

and further strengthens the concept that platelets bear significant structural and functional similarities to muscle cells. The functional importance of the electrical responses of the mammalian megakaryocytes is unclear, but supports the recent study by Fedorko⁵ demonstrating that megakaryocytes are contractile cells which respond to physiological agents in the same manner as platelets.

In the bone marrow, no direct associations are known between megakaryocyte and neurones, so that neuronal control of megakaryocyte activity would seem unlikely. Humoral agents, however, might regulate megakaryocyte function through changes in membrane potential. Activation of platelets by physiological agents might also effect potential changes resulting in a wave of membrane depolarisation and release of intracellular stores of calcium, subsequent contraction of platelet thrombosthenin, and ultimately platelet aggregation. Of interest in this regard is the report by Fantin *et al.*⁶ that electrical or mechanical stimulation promoted the retraction of *in vitro* clots by platelets, presumably also through the activation of the platelet contractile protein, thrombosthenin⁷. Further clues

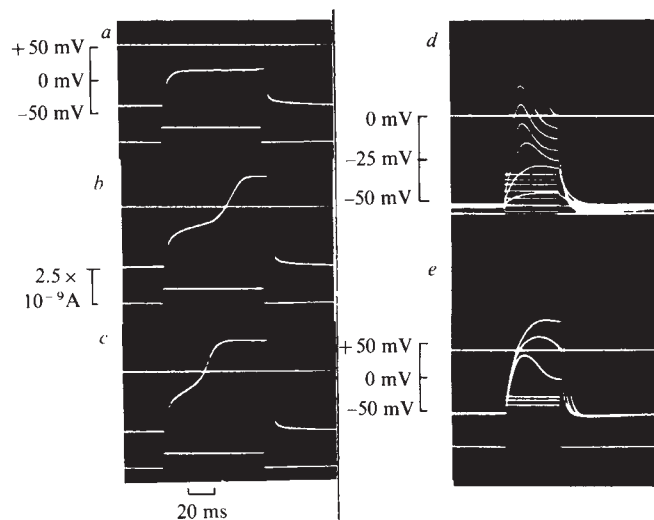


Fig. 4 *a-c*, Depolarising response requiring high intensity stimuli. At the apparent threshold for this cell (about +20 mV), equal current intensities produce passive depolarisation (*a*) or active depolarisations having variable delays (*b* and *c*). The medium contained 2.4 mM Ca^{2+} . *d* and *e*, Mixed response in another megakaryocyte showing graded character. Lower intensity stimuli (*d*) show progression from passive to active depolarisation. With higher intensity (*e*; note decrease in gain), the repolarising phase is abruptly lost and the second type of depolarising response is elicited. The medium contained 7.2 mM Ca^{2+} . Current and time calibrations apply to all traces. Note differences in voltage calibrations.

as to the physiological role of such responses in megakaryocytic cells must await more detailed electrophysiological investigations.

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Growth and membrane lipid properties of *Acholeplasma laidlawii* B lacking fatty acid heterogeneity

The lipids of biological membranes are generally highly heterogeneous in both head group and fatty acid composition. The membrane lipids of most organisms contain an assortment of fatty acids of various hydrocarbon chain lengths and configurations; the cytoplasmic membrane lipids of *Escherichia coli*, for example, contain saturated, *cis*-monounsaturated and *cis*-cyclopropane fatty acids of 14-19 carbons¹. To date, the role(s) of this fatty acid heterogeneity remains largely unclear. We report here

Table 1 Exogenous fatty acid enrichment and phase transition parameters for membrane lipids of cells cultured with CM-55 $5 \mu\text{g ml}^{-1}$ plus various fatty acids at 35°C , 28°C or 25°C

Exogenous fatty acid	Growth temperature ($^\circ\text{C}$)	% Enrichment	T_c ($^\circ\text{C}$)	$T_L - T_S$ ($^\circ\text{C}$)
14:0	35	85	ND	ND
16:0i	28	94	19	10
	25	96	19	10
16:1 Δ^9	28	93	13	8.5
	25	98	9	8
18:1 Δ^9	28	93	24	12
	25	98	22	11
18:1 Δ^{11}	28	86	<0	ND
18:1 Δ^{11}	28	87	24	10
18:1 Δ^6	28	80	22	16

Cells were cultured in a modified Edward medium supplemented with bovine serum albumin 4 mg ml^{-1} and glucose 2.5 mg ml^{-1} as described elsewhere¹. Fatty acid composition of the membrane lipids was determined by gas-chromatographic analysis of the derived methyl esters as described previously¹. Differential thermal analysis of the extracted membrane lipids was carried out as described previously⁵ after equilibration of the lipids with Mg^{2+} -containing buffer as described in the text.

ND, Not determined.

that by using an antilipogenic compound and by adding a single fatty acid to the culture medium of the simple cell wall-less procaryote *Acholeplasma laidlawii* B, it is possible essentially to abolish lipid fatty acid heterogeneity without any obvious effects on cell growth. Furthermore, the membrane lipids of cells cultured in these conditions show a greatly sharpened thermotropic gel-to-liquid crystalline phase transition, indicating that neither fatty acid heterogeneity nor a low-cooperativity lipid-phase transition are essential for proper membrane function in this organism.

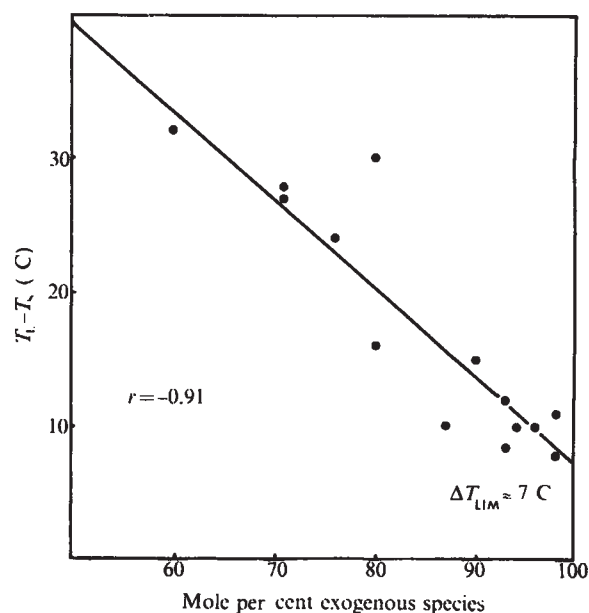
The antilipogenic compound *N,N*-dimethyl-*trans*-4-oxo-2-dodecenamide (CM-55) was first reported by Omura *et al.*² and was synthesised by us essentially as described previously^{2,3}. CM-55 strongly inhibits endogenous fatty acid production in *A. laidlawii* B at levels as low as $5 \mu\text{g ml}^{-1}$, as shall be detailed elsewhere (unpublished results). Cells cultured with various fatty acids plus $5 \mu\text{g ml}^{-1}$ CM-55 show pronounced enrichment of the membrane lipids in the exogenous species, typically to the extent of 85–95%, of total membrane lipid fatty acyl moieties at 35°C or 28°C and 95% (or more) at 25°C . At the latter temperature, the extent of lipid enrichment in the exogenous species is limited only by the incorporation of small amounts of contaminating long-chain fatty acids also present in the growth medium. As cell growth is markedly poorer at 25°C than at 28°C or 35°C , most experiments were carried out with cells grown at the higher temperatures; in a few cases, however, cells were grown at 25°C in carefully delipidated media to obtain membrane lipids of essentially homogeneous fatty acid composition. Several fatty acids (listed in Table 1) support cell growth rates and yields in the presence of $5 \mu\text{g ml}^{-1}$ CM-55 (determined turbidometrically as described elsewhere³) which are essentially identical to those of cells grown without CM-55. As shown in Table 1, all of these fatty acids were found to comprise greater than 85% of the total membrane lipid fatty acids when cells were cultured with CM-55; certain species were enriched to the extent of 98% (or more) at 25°C . A number of fatty acids, notably palmitate, stearate, laurate and linoleate, failed to support growth with CM-55 present at 35°C , suggesting that they fail to fulfill the physicochemical requirements for proper membrane function.

In order to assess the effect of a decreased heterogeneity of membrane lipid fatty acyl groups on the lipid physical properties, the lipids were isolated as described elsewhere¹,

equilibrated with a buffer of ionic composition similar to that of the growth medium (150 mM NaCl, 50 mM Tris, 5 mM MgSO_4 , pH 8.0) before re-extraction using the method of Bligh and Dyer⁴, and subjected to differential thermal analysis (DTA) as described previously. The lipid gel-to-liquid crystalline transition temperatures (T_c) and widths (corresponding to the difference in the temperatures of roughly 2% and 98% completion of the transition, which were the detectable limits of the transition in our apparatus) are given for several preparations in Table 1. The transition widths for the preparations closest to fatty acid homogeneity are $\sim 10^\circ$ as compared with 20 – 35° in samples derived from cells cultured without CM-55 and with or without exogenous fatty acids. When the transition width is plotted against the extent of enrichment of the membrane lipids in the exogenous species, using the data of Table 1 plus previously reported results for cells grown with these fatty acids but without CM-55, a strong correlation is observed ($r = -0.91$) between fatty acid heterogeneity and transition sharpness. This result indicates that the broadness of the normal membrane lipid phase transition ($\sim 30^\circ$) is due mainly to fatty acid heterogeneity (lauric, myristic and palmitic acids predominate in normal cells) and not to the diversity of lipid head groups. This result is particularly surprising in view of the fact that the *A. laidlawii* B membrane contains five major lipids (two glycolipids and two phospholipids, plus a phosphorylated glycolipid), none of which accounts for more than 35% of the total lipid in cells grown to late log phase.

Our results lead us to several interesting conclusions. First, it is clear that none of the normal fatty acids of *A. laidlawii* B (that is, straight-chain saturates of 12–18 carbons, plus 18 carbon *cis*-unsaturates) is required in the membrane in greater than trace amounts for optimal cell growth. Second, a marked sharpening of the membrane lipid phase transition does not diminish the viability of *A. laidlawii* B, even when the cells are grown at a temperature well above that of the phase transition. This observation suggests that the temperature- or ion-dependent lateral segregation of certain lipids into gel-state domains is not an essential membrane property, at least in this organism. Finally, as noted above, the normally low cooperativity of the membrane phase transition in *A. laidlawii*

Fig. 1 Correlation of lipid—phase transition width with extent of enrichment of the membrane lipids of *A. laidlawii* B in a single exogenous fatty acid. The fatty acid composition and the thermal phase transition of the membrane lipids were determined as described in Table 1.



B is due mainly to the heterogeneity of the fatty acyl chains and not of the head groups of the membrane lipids. We suggest that the reduced phase transition cooperativity which fatty acid heterogeneity creates may be of adaptive significance to the organism, giving rise to a less drastic change in membrane phase state in response to environmental perturbations than would occur were the transition highly cooperative. We have no reason to believe *A. laidlawii* B to be unique in this respect (recent studies with *E. coli* auxotrophs, for example, have yielded similar results⁸), and we suggest that this adaptive capacity may be a major function (possibly the major function) of lipid fatty acyl group heterogeneity in other biological membranes, particularly those which are low in sterols.

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Regulation of nitrate reduction by light, ATP and mitochondrial respiration in wheat leaves

ASSIMILATION of nitrate in leaves is closely linked with photosynthesis^{1,2} because ammonia, the end product of the former process, is incorporated into amino acids by means of carbon skeletons derived from the assimilation of CO₂. Canvin and Atkins^{3,4} have shown that the reduction of nitrate to nitrite in leaves of barley is dependent on light and ceases when the light is extinguished. On the other hand, in the *in vivo*⁵ method for assay of nitrate reductase, nitrate is reduced to nitrite by leaf disks even in the dark. As Canvin and Atkins^{3,4} pointed out, the *in vivo* method is not a true reflection of what happens in plants in physiological conditions. A regulatory mechanism must exist in leaves, which shuts off nitrate reduction immediately when light is extinguished, so that the accumulation of toxic amounts of nitrite, which can only be reduced by photosynthetic reactions⁶, is avoided. We now propose that this regulation functions through mitochondrial respiration, which operates in the dark, but is inhibited in light, because of the increased cytoplasmic adenylate charge due to photosynthesis.

Intact wheat seedlings or whole leaves from 10-d-old plants (grown in a phytotron with 10 mM KNO₃), when incubated in an oxygen-free atmosphere in the dark, reduced substantial quantities of nitrate to nitrite. This reaction was extremely sensitive to O₂, as shown in Fig. 1. Complete inhibition of nitrite formation was observed with 1.35 µg atoms of O₂, which is less than 1% of the normal O₂ concentration of air. Results in Fig. 2 show that nitrate was rapidly reduced, even in air, when intact leaves were treated with carbon monoxide, an inhibitor of the mitochondrial electron transfer chain at the cytochrome oxidase step. This carbon monoxide effect was readily reversed by light, which dissociates the cytochrome oxidase-CO complex. The nitrite which had already accumulated in the leaves in the dark was rapidly reduced by nitrite reductase, which functions only in light⁶. When

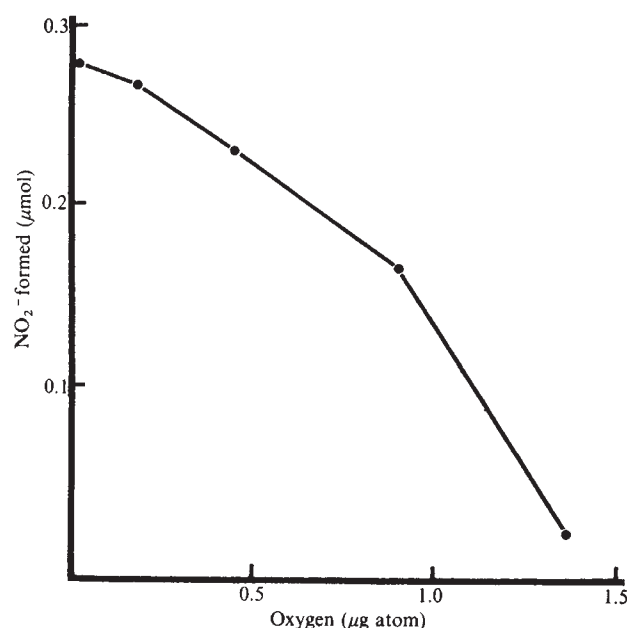


Fig. 1 Inhibition by O₂ of nitrate reduction in intact wheat leaves. Intact leaves (200 mg) were folded and placed in Warburg flasks. The flasks were evacuated thoroughly to remove air. Different amounts of air were then injected through subsials fitted to the sidearms of the flasks with an air-tight microsyringe to give the indicated concentration of oxygen. After 30 min at 30 °C in the dark, the nitrite formed in the leaves (µmol NO₂⁻ per 0.2 g tissue per 30 min) was determined as described in ref. 8.

these leaves were again transferred to the dark, nitrite was not produced because the cytochrome oxidase-CO complex had already been dissociated by the previous light treatment. Brief exposure of leaves to CO for 30 s, however, again substantially stimulated nitrite production in the dark. Inhibition of electron transport to O₂ by either amylal or rotenone also stimulated nitrite accumulation (Table 1). These inhibitors were vacuum-infiltrated into leaf disks in a liquid medium. In the control treatments without inhibitors, subsequent shaking in air inhibited nitrite production by half, which was equivalent to that observed for 1 µg atom of O₂ in Fig. 1 where the experiments were done with detached leaves in a gaseous phase. The extent of inhibition depends on the rate of diffusion of O₂ into the leaves, which is expected to be considerably less in a liquid medium.

The results in Figs 1 and 2 and in Table 1 thus show that the inhibition of electron transfer through the mitochondrial chain to oxygen, by anaerobic conditions or by inhibitors, triggers off the reduction of nitrate in the dark. If mitochondrial respiration is similarly affected by light, through the photosynthetic reactions, then the activation of nitrate reduction by light and its immediate cessation when light is extinguished can be readily explained. Such inhibition of dark respiration has been shown by numerous workers (for review see ref. 7). It has been shown that the light-dependent increase in the cytoplasmic ATP-ADP ratio, which is transmitted to mitochondria through the adenylate translocator, inhibits dark mitochondrial respiration, which is itself controlled by the adenylate charge. The energy derived from photophosphorylation in the chloroplasts is transferred to the cytoplasm in the form of triosephosphates, which generate ATP in the cytoplasm.

To examine whether raised concentrations of cellular ATP could promote nitrate reduction, adenine nucleotides and glycolytic intermediates were vacuum infiltrated into the leaf disks. The results in Table 1 (experiment 2) show that infiltration with either ATP or fructose-1, 6-diphosphate resulted in a significant accumulation of

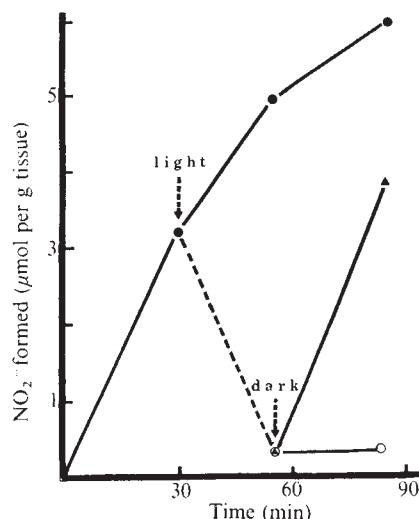


Fig. 2 Reduction of nitrate to nitrite in CO-treated leaves in the dark and its reversal by light. Intact leaves (300 mg fresh weight) from 10-d-old wheat seedlings (var. Halbred) grown with 10 mM KNO_3 , placed in 1.5 × 15 cm test tubes, were flushed with CO for 30 s and kept in the dark at 30 °C. After 30 min, the leaves were transferred to other unflushed test tubes. One batch was allowed to remain in the dark (●) whereas the other batch was exposed to light (20,000 lx) for 20 min (▲); then half of these plants were transferred to the dark (○) while the other half was again flushed for 30 s with CO before being placed in the dark (▲). At the indicated times, nitrite was extracted from the leaves by immersing them in 9 ml of boiling distilled water for 15 min. After cooling, the leaf material was macerated in a pestle and mortar and the homogenate centrifuged at 3,000 g for 20 min. Nitrite was determined in the supernatant fraction by the method of Hewitt and Nicholas⁸.

nitrite, even in the presence of air. A smaller stimulation of nitrite accumulation also occurs with ADP, but this could result from ATP generation by adenylate kinase in leaf disks. It is of interest that AMP and P_i had no effect. Klepper *et al.*⁵ reported stimulation of nitrite production in isolated leaf disks by infiltration with fructose-1,

Table 1 Effects of inhibitors of mitochondrial electron transfer chain, the adenine nucleotides and fructose-1,6-diphosphate on nitrate reduction by leaves in the dark

Additions to infiltration media	Concentration (mM)	$\mu\text{mol NO}_2^-$ produced per g tissue per h
Experiment 1*		
None (stationary)	—	1.74
None (aerated)	—	0.88
Rotenone	0.50	1.36
	1.25	1.59
	2.50	2.60
Amytal	1.00	1.43
	2.50	1.14
Experiment 2†		
None (control)	—	0.70
ATP	5	2.12
ADP	5	1.80
AMP	5	0.72
Fructose-1,6-diphosphate	10	1.46

*Experiment 1. 300 mg of 0.5 cm long leaf disks prepared from leaves of wheat seedlings grown as described in Fig. 1 taken in 20 ml Erlenmeyer flasks, were vacuum infiltrated with a medium containing 4 ml of 0.1 M Tris-HCl (pH 7.5), 1 ml of ethanol 90% (v/v) and either rotenone or amytal. In control treatments Tris-HCl and ethanol only was infiltrated. These were then transferred to the dark at 30 °C. One of the flasks, without inhibitor, was kept stationary whereas the remaining flasks were aerated on a rotary shaker (100 oscillations min^{-1}). After 60 min, the total nitrite produced was determined as described in Fig. 1.

†Experiment 2. The procedure was the same as for experiment 1 except that the leaf disks were placed in test tubes and infiltrated with 1 ml of either distilled water (control) or with the listed concentrations of the neutralised solutions of the metabolites. The tubes were then placed in the dark at 30 °C without shaking and after 45 min the nitrite produced was determined as described in Fig. 1.

Table 2 Effects of arsenate on nitrate reduction in leaf disks in the dark in the presence of fructose-1,6-diphosphate

Experimental conditions	$\mu\text{mol NO}_2^-$ produced per g tissue per h
Anaerobic	3.60
Anaerobic + arsenate	3.30
Aerobic	0.18
Aerobic + fructose-1,6-diphosphate	0.96
Aerobic + fructose-1,6-diphosphate + arsenate	0.12

Leaf disks (300 mg), placed in Thunberg tubes, were infiltrated with 1 ml of either 10 mM phosphate buffer (pH 7.5) or phosphate buffer containing fructose-1,6-diphosphate (6 mM) and/or arsenate (20 mM). After the final evacuation some tubes were incubated under anaerobic conditions and others in air, all in the dark. Nitrite produced after 30 min at 30 °C was determined as described in ref. 8.

6-diphosphate. They attributed this effect to the production of NADH by glyceraldehyde-3-phosphate dehydrogenase during glycolysis. Since ATP is also produced in the same reaction, however, the observed stimulation could result from the action of ATP rather than NADH. To examine this possibility, leaf disks were infiltrated with arsenate, which prevents the formation of ATP in this enzyme system without affecting the production of NADH. Arsenate completely reversed the stimulatory effect of fructose-1,6-diphosphate on nitrate reduction (Table 2). It is therefore, ATP produced from fructose-1,6-diphosphate which stimulates nitrite production in air, thus confirming the data in Table 1.

The observation of Calvin and Atkins^{3,4} that the reduction of ^{15}N -labelled nitrate to nitrite in leaves is strictly light dependent and ceases abruptly when it is extinguished can now be explained on the basis of inhibition of mitochondrial respiration by photosynthesis. Thus the cytoplasmic adenylate charge which is increased by photosynthesis inhibits the mitochondrial electron transfer chain from NADH to oxygen. This accumulated NADH is then used for the reduction of nitrate.

In light, when sufficient ATP is produced by photosynthetic reactions, this nucleotide is not generated by the respiratory electron transfer chain. In these conditions, nitrate reduction is activated and the nitrite produced would then be converted to ammonia in the chloroplasts, with reduced ferredoxin acting as the reductant⁶. When light is extinguished, mitochondrial respiration takes over and NADH is oxidised by way of the respiratory chain: thus NADH is not now available for nitrate reduction.

Thus light, ATP and mitochondrial respiration together produce a very elegant regulatory mechanism for the reduction of nitrate to nitrite in green leaves.

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matters arising

Has the Sun a companion star?

HARRISON¹ suggests that the Sun is accelerating at $\sim 10^{-6}$ cm s⁻² in the general direction of the galactic centre, and attributes this acceleration to a companion star with

$$M/R^2 = 1.7 \times 10^{-6} \quad (1)$$

where M is the mass in solar units and R is the distance in AU. Analysis of planetary perturbations² gives a limit on the tidal acceleration equivalent to

$$M/R^3 < 2.7 \times 10^{-11} \quad (2)$$

Combining equations (1) and (2) gives

$$R > 6 \times 10^4 \text{ AU} = 0.3 \text{ pc} \quad (3)$$

and

$$M > 6 \times 10^3 \quad (4)$$

The orbital period is $> 1.8 \times 10^5$ yr and the velocity is > 9 km s⁻¹.

If we accept a supermassive black hole as the solar companion, it will have observable consequences. The gravitational deflection of the light from a star 1 arc min from the black hole is > 3 arc s. The black hole moves < 6 arc s in six months due to parallax and < 7 arc s yr⁻¹ due to orbital motion. This motion will modulate the gravitational deflection, leading to a detectable pattern of proper motions and parallaxes in the background stars.

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HARRISON REPLIES—I am not competent to judge the analysis by Rawlins and Hammerton of Neptune's residuals. I notice that these authors exclude the possibility of a perturbing object at distances greater than 300 AU, and assign a probable longitude of $319^\circ \pm 24^\circ$ to a hypothetical tenth planet that is roughly 55° from the suggested companion star. My impression is that it is not easy to determine with precision the residuals of an orbit that has been

observed continuously for a time less than one period (in this case 165 yr) when the disturbing force has a very much longer period ($\sim 10^4$ yr). Also, if the companion is temporary only, and is a neutron star moving at the high velocity typical of such objects, it would pass by the Solar System in a time of 50–100 yr. Neptune's residuals in this case, if determinable, might then be small, and even smaller, if the neutron star is moving toward the Solar System.

It has been suggested^{1,2} that condensations in the early stages of the Galaxy may have resulted in the formation of numerous supermassive black holes of masses 10^3 – $10^6 M_\odot$. Wright's comment on the possibility of a supermassive black hole at a distance of one light year is therefore interesting. Such an object in Sagittarius, or thereabouts, should be identifiable from radiation emitted by the infalling interstellar medium.

The hypothesis of a companion star has the virtue of being falsifiable (using Popper's terminology), and in my opinion the easiest way at present to falsify the hypothesis is to find a pulsar of an anomalous period-derivative in the opposite hemisphere of the sky.

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Absolute radiocarbon dating by low-altitude European tree-ring calibration

PEARSON *et al.*¹ recently reported the results of 58 precision radiocarbon measurements of samples from a floating tree-ring chronology 1,140 rings long, essentially covering the third millennium BC. Unfortunately, the authors chose to approximate their results by a straight line, obscuring the most interesting part of their results, the so called 'wiggles' in the functional relationship between radiocarbon years and calendar years. There seems to be a psychological preference for a smooth linear relationship, although such a relationship cannot be considered *a priori* more probable than an irregular one². Pearson *et al.* conclude that the fact

that a linear relationship exists, which approximates within limits of errors their experimental data 'rules out over the period investigated, the use of wiggle matching as a dating technique.' This dating technique has been used successfully not only by the La Jolla Laboratory³ but also by the laboratories in Groningen⁴, in Bern⁵, and possibly elsewhere. Its applications included the period under consideration. Also, Pearson *et al.* do not seem to be aware of the fact that hundreds of measurements have shown that within experimental errors the wiggles in the calibration curve are identical in the California bristlecone pine and in oak trees from central Europe^{6,7}.

The most deplorable fact concerning 'smoothed calibrations' as derived by a number of authors^{8,9} is that they give the erroneous impression that irregularities are essentially caused by experimental errors. Such a misconception, however, leads to a gross underestimation of the intrinsic errors necessarily incurred in calibration. The article by Pearson *et al.* tends to support this misconception and the purpose of this note is to correct it.

I have presented elsewhere the numerical results of some 700 ¹⁴C measurements of tree ring dated wood carried out so far in La Jolla¹⁰. A statistical analysis and discussion of these results will be published later. Sixty-two of them came from the period under consideration. The measured samples were bristlecone pine wood that had been tree-ring dated by C. W. Ferguson of the University of Arizona. Also included were the results of 55 samples from the same period of time which consisted of wood samples from a floating 1,250-ring chronology, the Becker Bronze age chronology of European oak¹¹.

The agreement in the features obtained in the three series is indeed remarkable. Certainly Pearson *et al.* have shown that by applying the necessary care and precautions, their scintillation counting technique for radiocarbon measurements can compete successfully with any gas counting system presently in operation. The authors, however, seem to have underestimated the accuracy of their own results or else they would have noticed that an irregular curve of the type of my calibration curve of 1969 would better approximate their results than the straight line that they propose. This can be seen from the way in which the data points cluster alternately on one side of the regression line and then on the other (Fig. 1 in ref. 1).

To what an extent my 1969 curve will require corrections and improvements was discussed previously.^{6,7} For the third millenium BC, the period covered by the Belfast measurements, it can be seen that the wiggle indicated on my early curve for the twenty-second century BC does not exist. The character of the rest of the calibration curve, and in particular its stepwise character, however, is well represented by all the more recent measurements.

Professor Ferguson has supplied some 20 additional bristlecone pine wood samples from the period under consideration and these samples are being measured at present. As soon as these, and some additional measurements are completed, I intend to publish a paper that will discuss the results in detail and will propose a revised calibration curve for the third millenium BC.

Operation of the La Jolla Radiocarbon Laboratory is financially supported by grant NSF EAR76-22623 from the Earth Science Division, Geochemistry Section, of the USNSF.

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PEARSON REPLIES—I feel that to reply in detail to Suess's comments at this stage before his recent dates, statistical analyses and discussion are in print, would be of little value. It would be helpful in later discussion if Suess gives details of his experimental technique, and any corrections used to evaluate the radiocarbon dates together with relevant information which will justify drawing any curve other than the most simple, especially if the latter is as good a fit to the data as is the case presented in our article.

I have re-examined the data presented in our article and my findings are consistent with those published in that the precision on a single measurement is ± 25 yr standard deviation and the linear regression gives value for $r = 0.99$ and F is approximately 3,000 giving a very good fit to the experimental data. However, as

stated in the article (see discussion) fluctuation can still exist within a 3% limit and this is supported by P. E. Damon and J. C. Lerman in their detailed statistical analysis of the data (personal communication).

If 'wiggles' of this magnitude are to be reproduced and matched from three different sets of data, then I feel more information is required from Suess before more detailed discussion can take place.

We sincerely thank Suess for his congratulatory comments on the quality of measurement and for raising the points commenting on our paper. I reserve judgement on the relative quality of gas to scintillation counting techniques since both are in operation in this laboratory and are now being compared. We await with interest the publication of his methods and statistical analysis justifying any deviation from the derived conclusion of a straight line fit to the data as shown in our article.

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Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

Anomalous LF radiowave records associated with meteoric ionisation

Information on the ionic constituents of meteor trains and the residual ions in the lower thermosphere is sparse. Sen and Saha¹ made the interesting suggestion that low frequency (LF) radiowave observations could provide information on meteoric ions and diffusion processes and in particular that the field strength characteristics of a 280 kHz navigational

beacon were dependent on absorption by meteoric ionisation. Though such ionisation was suggested as being responsible for the anomalous signal characteristics it seems unlikely that this interpretation is correct.

A column of meteoric ionisation situated below the LF signal daytime reflection height (~ 90 km) must, in order to yield the reported attenuation of up to 8 db, provide substantial absorption over an area comparable in size with a fresnel zone (~ 10 km). Thus, for a meteor train of gaussian radial distribution (length ~ 10 km and radius ~ 5 km) situated along a ray path to provide an absorption in excess of 1 neper over the column cross-section, requires an axial electron density greater than about $2 \times 10^3 \text{ cm}^{-3}$ (train 70-80 km) or $8 \times 10^3 \text{ cm}^{-3}$ (train 80-90 km) (assuming an electron collision frequency increasing from $1.2 \times 10^5 \text{ Hz}$ at 90 km to $3.4 \times 10^6 \text{ Hz}$ at 70 km). The corresponding meteor train electron line densities α (cm^{-1}) are 2×10^{15} and 7×10^{15} and for a more general geometry these values certainly represent minimum requirements. The initial radii of large meteor ionisation columns are a few metres. Train expansion cannot be faster than that expected from eddy diffusion (coefficient $> 10^6 \text{ cm}^2 \text{ s}^{-1}$) so the minimum time to achieve a train radius of 5 km is 17 h (about 2 orders of magnitude greater than the reported t_r values¹). Further, it is well known that severe meteoric ionisation loss occurs below 90 km, HF radio-meteor echo durations only very rarely² exceeding 10²s. Such characteristics² are in good accord with theoretical models³ which show that for large meteors the ratios α/α_{1-0} after only 10³ s are 2×10^{-2} and 3×10^{-4} at 85 and 75 km respectively. Clearly quite unrealistically large meteors incident in a critically small volume of the atmosphere are required to produce 474 absorption events yr^{-1} .

We may enquire as to the nature of the linear plots in Fig. 2 of ref. 1 which are attributed to the effects of ion diffusion. The lines have gradients of 1, $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{4}$, $\frac{1}{5}$, and $\frac{1}{6}$. Since this gradient is actually $1/t_r$, it seems very likely that the different lines (corresponding to $t_r = 1, 2, 2.5, 3$ s and so on) simply represent the details of data reduction and analysis.

Thus the interpretation of Sen and Saha¹ is quite incompatible with known meteor characteristics.

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reviews

Forms of mental phenomena

Bernard Meltzer

Artificial Intelligence and Natural Man. By Margaret Boden. Pp. 533. (Harvester Press: Sussex, 1977.) Hardback £13.50; paperback £4.95.

ARISTOTLE, I believe, characterised form as being "all of the boat that is not the wood". Artificial intelligence may illuminatingly be looked upon as the study of all of thinking that is not physical process. To understand and make use of form, we have to resort to representations of it—for instance, in the case of the boat, to a sketch, a blue-print or a verbal description. The best and most pregnant kind of representation man has yet found for exhibiting the forms of thought is a computer program. This is due firstly to the apparently limitless precision and power of programming languages for the description of processes of any degree of complexity and at any level of detail. But, more importantly, it is due to the fact that such a representation, though not that of a physical process, can be imposed upon and direct the physical processes of a computer, generating intelligible behaviour. Thereby, the criterion of science can be applied to such a representation: does it work? One cannot sail in the blue-print of a boat, but one can, for example, converse on some subject matter with a successful natural language understanding program, or one may have a program—on a computer provided with suitable sensor and motor peripherals—carry out in a versatile way many assembly tasks.

The main content of Dr Boden's book is a careful account and examination of a selection of representative pieces of work that have been done in trying to tease out the forms of mental phenomena like the understanding of natural language, visual perception, belief systems, neurosis, learning, problem solving and creativity. It is one of a rapidly increasing number of introductory accounts of the subject being published, the two best until now probably being American: Winston's *Artificial Intelligence* and Raphael's *The Thinking Computer*. These and Dr Boden's are genuinely introductory, in that they assume in the reader not only no knowledge of their subject but even no or hardly any knowledge of computers and how they work; the three differ in the amount of the latter included.

What mainly distinguishes the present book, however, is that it presents the view of an outsider, rather than a practitioner, of the discipline. This is responsible for its strengths, and for weaknesses to a lesser degree. The author's background of academic philosophy and psychology, as well as her humanist outlook, not only helps her not to let techniques rather than ideas dominate the presentation, but also is presumably responsible for its careful, critical and comprehensive scholarship, of a kind very rare in a brash, new, vigorous subject of this kind. The claims that are and might be made on behalf of any of the many cognitive models she describes are usually cut down to size, and often made the more convincing and impressive by that; and as for reading, the 34 pages of notes on the text in small print at the end of the book are a mine of information to guide one in the literature of and around the subject.

The writing is very clear and often elegant, though occasionally pedagogical to the point of coyness; but though the contents of the pieces of work she describes are so well presented, little of the excitement that may accompany the exploration of new intellectual territory is conveyed. The organisation of the expository part of the book is what is called in the jargon of the subject "top-down"; thus, the account starts with Colby's simulation of neurosis, reaches vision programs about half-way through, and reserves some account of modern "non-deterministic" programming languages only to the very end. There is some lack of historical perspective: for example, I think neither Leibniz nor Babbage is even mentioned.

But the book has a secondary purpose besides exposition. To quote from the preface: "I have tried to convey a sense of the relevance of artificial in-

telligence to the understanding of natural man. Contrary to what most people assume, this field of research has a potential for counteracting the dehumanising influence of natural science. . . . The common view that machine research must tend to display us humiliatingly to ourselves as "mere clockwork" is false. The more widely this is realised, the less of a threat will artificial intelligence present to humane conceptions of society." Although one may strongly doubt that "dehumanisation" in our society is due to the influence of natural science, Dr Boden does argue her general point well. This is done both implicitly in the main expository part of the text (accounting—apart from its pedagogical advantage—perhaps for the top-down approach referred to above), and in a final section confronting directly psychological, philosophical and social questions raised. This section ranges widely and interestingly, for example, over the mind-body "problem", possible limitations on the computational representation of thought, aspects of the modelling of emotion, possible beneficial applications in education, and other applications elsewhere.

Boden is probably too conservative in her assessment of the likely effect on our culture of the development of artificial intelligence. And she incidentally answers in some detail many of the criticisms voiced by Dreyfus and others hostile to the subject. For example, she shows how tasks confidently claimed by some of the critics to be beyond the power of computational representation, like the understanding of metaphor or the use of what Polanyi called "tacit" knowledge, are being broached in existing programs. □

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Handbook of human evolution

Guide to Fossil Man. (Third edition.) By Michael H. Day. Pp. xviii+346. (University of Chicago Press: Chicago; Cassell: London, 1977.) \$15; £7.95.

THE fossil evidence for human evolution has been accumulating for almost a century and a half, and throughout most of this period the remains of fossil man

have appeared in a slow, if not leisurely, procession. Thus, the slowly emerging record of human evolution could easily be assimilated by specialist, student and layman alike. However, beginning about 1960 the study of fossil man entered a period of remarkable and dynamic productivity. Within the past 18 years more than 500 new hominid fossils have been

added to the known record; the majority of these have, in fact, been added in the past seven years. Not only is the sheer volume of the new material staggering but many long-cherished theories and dogmas have been challenged, if not discredited. Thus, even specialists now have a difficult time keeping abreast of newly discovered fossils, new sites and new interpretations. Michael Day is an exception, however, as the third edition of the *Guide to Fossil Man* clearly shows.

As in the earlier editions of the *Guide*, Professor Day has set a style as remarkable for its brevity as for its content. The basic data of about 40 fossil sites is conveyed in concise yet readable paragraphs. Data on taxonomic (or vernacular) synonyms, dating, faunal and archaeological associations, taxonomic affinities and morphology, are quickly available. A comprehensive bibliography accompanies each site entry and most of the fossil hominids are well illustrated. The sites included have been selected for their interest and importance from a large number of possibilities. Thus, although the *Guide* is less complete than the *Catalogue of Fossil Hominids* (reviewed in *Nature*, 270, 766; 1977) it is of more immediate and broader interest than those encyclopaedic volumes.

Although the format of the *Guide* is concise, a considerable amount of information is conveyed in the categories of morphology and phylogenetic affinities. These sections are particularly informative and useful, as Professor Day, unlike many authors of books on fossil man, has personally examined much of the original material. Such first hand acquaintance with the fossil evidence considerably enhances the authority of these sections.

Although designed as a source book, rather than as a text, the format and content of the *Guide* nevertheless allows it to be used successfully in the classroom. Experience with the earlier editions has indicated that students appreciate the easy availability of descriptive and historical data and the accessible bibliographical information. The expanded summary areas, covering the Neandertal 'problem' and the affinities of the australopithecines as well as listings of the new material, has augmented these strong points.

All in all, the *Guide to Fossil Man* is a useful and readable handbook for anyone interested in human evolution.

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Birds of the Western Palearctic

Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic. Vol. 1: Ostrich to Ducks. Edited by S. Cramp, K. E. L. Simmons, I. J. Ferguson-Lees, E. M. Nicholson, M. A. Ogilvie, P. J. S. Olney, K. H. Voous and J. Wattel. Pp. 722. (Oxford University: Oxford, London and New York, 1977.) £25.

THIS volume is the first of seven planned to cover the birds of the Western Palearctic (in spite of its cumbersome title, the work has been known as BWP for the many years of its planning and gestation). As such it follows a very illustrious predecessor, *The Handbook of British Birds* (Witherby *et al.* 1938–41). Although there were later editions of the work, the changes were relatively minor. The Witherby *Handbook* has been, and indeed still is, the standard work for many western European countries. No other work has replaced it, though a Swiss work (*Handbuch der Vogel Mitteleuropas* by Bauer and Glutz, 1966 *et seq.*) is currently in progress.

Plainly, the Witherby *Handbook* has become progressively more out of date; the work under review is intended to replace it. The main alteration in design is to include a greatly increased area of the Palearctic, from roughly 20°N to 50°E (Greenland is excluded). The choice of such a southern limit, although conform-

ing with certain other authors, involves the work with a few poorly known north African species (such as the Ostrich) which are hardly part of our avifauna; one slightly wonders why a more northerly southern limit, such as the edge of the Sahara, was not chosen.

This work, as the previous one, is the product of a team of workers, British and Dutch. Each of the 122 species covered in this volume gets a section to itself. These average about 4–12 pages of text, though a few vagrants which have not occurred recently get much shorter treatment. For each species, separate sections cover Field Characters and Habitat, Distribution and Population, Movements, Food, Social Pattern and Behaviour, Voice, Breeding, Plumages, Molt, Measurements, Weights, Structure and Geographical Variation. The text is well documented with key references.

Also, for each species, except the vagrants, there are normally two maps and two diagrams. The two maps cover the world distribution and, on a larger scale, the distribution within the Western Palearctic. The latter shows summer and winter distributions in red and grey, respectively. These are not always very clear; small pockets of wintering ground, grey on a basically grey map, are difficult to see and the seriously colour-blind may have trouble distinguishing red (summer) from red on grey (resident all the year round). The two diagrams are a voice sonogram and a clockface type of diagram, showing patterns of migration,

breeding, moult, and so on, for the species throughout the year. Personally, I find this rather too small to read easily; in addition, it has to be read with care, as the seasonal activities of a given species may be quite different in, for example, North Africa and Scandinavia.

Each species is depicted in colour, showing the bird in different plumages where distinguishable (summer, winter, male, female, juvenile, and so on). The 108 plates are by a number of different artists and not all are equally pleasing to my eye: many are packed rather tightly with birds; in particular, I find the downy young of some of the shearwaters and petrels unsatisfactory. In addition, the text often includes line drawings of the birds in behavioural postures.

The above gives, hopefully, an adequate description of the book and its contents. It is perhaps worth considering the value of such a book, particularly as, when complete, it is going to be an expensive work. Handbooks, by their very nature, are summaries of the available literature; a source of quick information or a starting point for further searches. Whole monographs or handbooks exist for many of the taxa of birds or even species dealt with in this volume; this is especially true for the wildfowl on which there is an enormous literature. Thus, of necessity, the writing of a handbook becomes an exercise in condensation: as time goes on this becomes increasingly more difficult; more has to be omitted. Certain of these difficulties are apparent when comparing this work with the earlier *Handbook*. Although larger, the text seems more compressed, more turgid.

However, the complaints are very minor; the present volume provides a wealth of information which is available nowhere else except in extensive libraries. There simply is no serious rival for the English reader. If one was prepared to be satisfied with much simpler information, I would recommend *The Popular Handbook of British Birds* (Hollom, latest edition 1977, £5.50), a single volume based on the Witherby work. For greater detail there is no alternative. The Swiss work mentioned above would be a serious rival for German-speaking ornithologists, but despite its qualities, the latest volume (no. 7 of 13) costs about £45.

One can be confident, therefore, that this work will become a standard reference. Although the physical production of the review copy is open to some criticism (the tops of some plates are cut off and the spine seems weak), the book is otherwise well produced and pleasingly free from errors. It contains a wealth of information and is a worthy successor to an illustrious ancestor. C. M. Perrins

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Herbal history

An Illustrated History of the Herbals. By F. J. Anderson. Pp. 270. (Columbia University Press: New York, 1977.) \$21.20.

A HERBAL is a book about the plants and other materials used in medicine up to the middle of the seventeenth century, though their use lingered on after that. *An Illustrated History of the Herbals* is an interesting account of these works (in many cases compilations) which most people never have a chance of seeing, locked up as they are in libraries. The words 'locked up' are used advisedly, as herbals are among the most precious of all books, not only because of their rarity but because they include some of the earliest examples of the printer's art.

Many of us have had recourse to Gerard or Parkinson at some time or other, but few go further back, even to Turner or Fuchs, though we may be aware of the earlier works and have access to some of them. Mr Anderson's book is all the more valuable for opening up this lost world, alien though it may be. Little of what was written in the mediaeval herbals relied on direct observation and there is a good deal of mumbo-jumbo, not that either of these defects has entirely disappeared from all modern books on medicinal plants. Indeed, it is salutary and somewhat chastening to note how little has changed in some respects: though astrology is no longer called in to help with the times for



Illustrations taken from *An Illustrated History of the Herbals*, reviewed on this page. Left, John Gerard, from the first edition of his *Herball*, published in 1597. Gerard, superintendent of the gardens of William Cecil (Lord Burghley), holds a potato—supplied to him for his garden (because of his connections with Raleigh and Drake) long before it became a common item. Right, Rembert Dodoens, from the English version of his *Herbal*. Dodoens' work on plants of The Netherlands formed the basis for the later systems of Cesalpino, Bauhin and Linnaeus, and his *Nievue Herball* (1578) may well have supplied Shakespeare with much of the plant lore used in the plays.

seed sowing or to diagnose illness, many people still say they believe in horoscopes!

The gradual realisation that the plants mentioned in Dioscorides were not necessarily the same as those in northern Europe, the various attempts at a system of classification, and the increase in scientific knowledge, these may all be seen in the herbals as they came to an end, to be replaced by works on botany and separated from medicine; the science of

botany has its roots in the past of the herbals, and owes them a great debt.

Mr Anderson's book is well produced and delightfully illustrated, but some errors have crept into the captions, which seem to be rather less scholarly in style and content than the rest of this charming book.

Rosemary Angel

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Function of zoological illustration

Zoological Illustration: An Essay Towards a History of Printed Zoological Pictures. By David Knight. Pp. 204. (William Dawson: Folkestone, UK, 1977.) £10.

In some natural sciences, results and ideas can be communicated successfully in wholly verbal or mathematical languages, and the absence of illustrations may even be taken as a mark of the science's maturity. In other sciences, visual representations remain indispensable, however 'mature' the field becomes. But their cognitive and social functions, and therefore their style, may change greatly in the course of time. For example, ostensibly theory-free and 'realistic' pictures are often replaced by increasingly diagrammatic representations, which can only be interpreted by those trained in the visual conventions of the discipline: the highly abstract diagrams of organic chemistry and tectonic geology are

good examples. At the same time, more 'realistic' illustrations may continue to flourish as means of visual communication on a more popular level, or to satisfy less cognitive and more aesthetic demands.

The history of zoological illustration would make a fascinating case-study of the changing role and style of visual communication in a science in which pictures of some kind are virtually indispensable. It is sad that Knight's book misses this opportunity. He rightly poses the question, "what function in science have zoological illustrations played at different times?", but the answers given are too diffuse to be of much use. Some of the best insights, such as the comment that "one cannot draw what one does not understand", are given as throw-away passing remarks, instead of providing an interpretative framework for the whole work.

The text contains many interesting items of information, but its style can only be described as rambling and repetitive. Most surprisingly for a work on this subject, the illustrations reproduced are not tied into the text by any cross-referencing. There is a useful

survey of the various techniques of reproduction, but their effects on visual communication are not evaluated systematically, and the illustrative examples are not identified in the captions as 'woodcut', 'copper engraving', 'lithograph', and so on.

The illustrations surveyed range in time from the beginnings of printing to about the mid-nineteenth century (plus one incongruous electron micrograph), with a heavy emphasis on the decades around 1800 and on the more ornamental or 'coffee-table' kind of book. Both text and illustrations are also rather strongly weighted towards British sources and the history of British science.

As the aesthetic impact of the great nineteenth-century colour-plate books is rightly emphasised in the text, it is sad that the reader is given only black-and-white reproductions of them, and rather muddy ones at that; surely at the price demanded the publishers could have allowed at least one coloured plate for a frontispiece.

Martin Rudwick

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Cellular biology of neurones

Handbook of Physiology: A Critical, Comprehensive Presentation of Knowledge and Concepts. Section 1: The Nervous System. Vol. 1: Cellular Biology of Neurons, Parts 1 and 2. Edited by J. M. Brookhart, V. B. Mountcastle, E. R. Kandel and S. R. Geiger. Pp. 1-717 and 719-1182. (Williams and Wilkins: Baltimore, Maryland, 1977.) \$135.

It is almost 20 years since the American Physiological Society began publication of its *Handbook of Physiology* with three volumes on Neurophysiology. A much needed second edition now makes its appearance with a two-part volume on the Cellular Biology of Neurons. The books were written "to be useful to graduate students and scientists in other fields", and as a result the individual chapters form unusually complete accounts of their various topics, combining the virtues of textbook and review.

The volume opens with a magisterial account of the general morphology of neurones and neuroglia by Palay and Chan-Palay. Synaptic ultrastructure is described in greater detail with some striking freeze-fracture electron-micrographs in the chapter by Heuser and Reese.

The section on excitation and conduction contains an extensive account by Rall of core conductor theory, an understanding of which is essential to an informed interpretation of intracellular potentials. Extracellular potentials are mentioned by Rall, and considered in slightly more detail by Shepherd in his chapter on the olfactory bulb, but the topic does not receive a quantitative treatment in the Handbook, an omission which many will regret. Hille's account of the ionic basis of resting and action potentials is easy to follow, authoritative and reasonably complete, though two important recent developments, the detection of gating currents and the application of noise analysis to excitable membranes, are only briefly mentioned. Constantine covers complementary ground in his chapter on the activation of striated muscle, and is to be congratulated on maintaining his composure while describing the Byzantine complexities of the ionic currents underlying the cardiac action potential. The chapter by Finkelstein and Mauro on physical principles of electrical excitability is among the best in the book, containing a long, hectoring and often brilliantly illuminating account of the thermodynamic basis of the membrane potential.

The section on junctional trans-

mission is the longest in the book, with 12 chapters devoted to structure (Heuser and Reese), postsynaptic mechanisms (Takeuchi), presynaptic mechanisms (Martin), electrical transmission (Bennett), smooth muscle and the autonomic nervous system (Holman and Hirst), a group of five chapters on specific transmitters (acetylcholine, the monoamines, and amino acids), neurosecretion (Mason and Bern), and axonal transport (Grafstein). Of these, Bennett's contribution makes the liveliest reading.

The article by Takeuchi in this section is seriously flawed by its imperfect English, something which should have been put right in the editorial office.

Part 2 is concerned with interactions between cells, with chapters on cell culture (Fischbach and Nelson), trophic interactions between nerve and muscle (Rosenthal), specificity of neuronal connections (Grinnell) and glial cells (Orkand) in the opening section. In the next section, Burke and Rudomin provide a comprehensive review of spinal neurones; Shepherd considers the olfactory bulb, with its curious reciprocal dendrodendritic synapses, as a model system for mammalian brain; and in a review of ambitious scope, the late Alden Spencer discusses the physiology of supraspinal neurones.

The final section of the book concentrates on invertebrate nervous systems, with chapters on the organisation of motor systems (Kennedy and

Davis), sensory systems (Wiersma and Roach), and neuronal plasticity and the modification of behaviour (Kandel).

In the final chapter, Kandel reviews recent evidence from his laboratory that habituation of the gill withdrawal reflex in *Aplysia*, which can last for a day or more, is due to a reduction in the mean number of quanta of transmitter released from sensory nerve terminals on to the gill motor neurones. Granted that habituation is a very simple form of learning, these experiments nevertheless come close to providing the beginnings of a physiology of the engram.

Considered as a work of reference this book has its faults. The most serious is the inadequacy of the Index, which contains too few entries with any practical value and too many with none. Cross-indexing in the text is perfunctory, and is to chapters rather than to specific pages. Some but not all chapters have summaries. Reference lists are remarkably complete up to 1975-76.

All in all, this is a magnificent piece of scholarship; the American Physiological Society has put all neurobiologists greatly in its debt. Librarians and those for whom moral debts are more irksome than the kind bank managers write letters about will want to buy their copy at once.

T. V. P. Bliss

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Rockslides and avalanches

Rockslides and Avalanches. Vol. 1: Natural Phenomena. Edited by B. Voigt. Pp. 833. (Elsevier: Amsterdam, New York and Oxford, 1978.) Dfl.240; \$98.

THE study of rockslides began in Switzerland with the Elm event of 1881 in which a rockslide buried the hamlet of Untertal, destroyed part of the village of Elm and killed 115 people. The canton authorities sent Albert Heim to investigate and he was surprised to discover that the rock debris had actually flowed considerable distances, rather than just fallen.

Of the various descriptive German terms which he used Hsu proposes that 'sturztrom' be adopted to denote slides of the type that affected Elm. Analysis of the Elm sturztrom by Hsu has shown that the accepted ideas of air-cushion movement, as developed by R. L. Shreve for the Blackhawk slide, are now rather suspect.

The first part of a two-volume survey of rockslides and avalanches, this book

deals with such natural phenomena. Volume 2 will contain the articles relating to engineering problems.

Besides the chapter dedicated to Heim and an introduction by B. Voigt and W. G. Pariseau, volume 1 contains sections on classic rockslides and avalanches (chapters 2-9); large-scale prehistoric mass movements (chapters 10-16); rock creep, scale effects and related questions (chapters 17-21); and mass movement of snow and ice (chapters 22-24); 833 pages costing £50—and no index; that will be found in volume 2.

The Blackhawk event gets an interesting chapter to itself, as do 13 other rockslides, and these chapters provide a very useful and readable set of histories. The apparent awkwardness of the sudden jump to principles, and to snow and ice problems, is probably caused by the fact that this is only half the complete work. The completed survey should be a valuable reference work but will presumably be beyond the means of individual buyers.

I. J. Smalley

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obituary

J. N. Mills

JOHN NORTON MILLS, Brackenbury Professor of Physiology at Manchester University, died in a tragic climbing accident in Snowdonia on 3 December 1977, at the age of 63. Born into a medical family, he was educated at Winchester and Oxford, and graduated in medicine at Birmingham in 1939. Following a brief interlude in general practice, his personality and talents directed him back to the more congenial cloisters of Oxbridge. After a Lectureship at New College, Oxford (1941–46), and a Lectureship and Fellowship at Jesus College, Cambridge (1946–1950), he departed for Manchester, as Lecturer in Human Physiology, where he spent the rest of his working life. Promoted Senior Lecturer in 1955 and Reader in 1959, he was appointed to the Brackenbury chair in 1965.

At Manchester, his earlier interests in respiration and the kidney converged into a wide programme of work on the components and mechanisms relating to human circadian rhythms and the 'internal clock'. In addition to a wealth of observations concerning the changes in rhythms associated with jet-travel, shift-work, and cavers living in isolation for extended periods—all of interest to the popular media—the establishment of an 'isolation unit' at Risley enabled John Mills and his colleagues to perform careful studies on humans living in experimental, controlled isolation. Fortunately, a series of definitive papers prepared during a sabbatical year, 1976–77, is now in press.

In the field of human chronobiology his contributions to books and journals were extensive; and from his position as a highly respected world authority, he was particularly involved in the activities of chronobiological journals and societies. From his deep interest and skills in field botany and climbing, the Linnean Society and various climbing clubs have known his writings. And the Physiological Society, Renal Association and Journal of Physiology have all benefited from his participation.

At Manchester, he was an early, effective campaigner for broadening representation on university bodies, and for the associated revision of the University Charter. His tenure as a founding co-editor of the university staff magazine was noted by brave encouragement (and, occasionally, pseudonymous authorship) of humor-

ous, satirical, occasionally scandalous articles, in the best polemical tradition, causing delight or offence in the reader according to personal opinions and prejudices. His occupancy of the Brackenbury chair was associated with the planning and development of the new medical school, and with the transformation of a small dilapidated department into one of the largest and best-equipped in Britain. After such a revolutionary period of change in structure and attitudes, he might have looked forward to a period of consolidation, and a strengthening of those aspects of the university in which he believed deeply—as a community of equals, providing education, culture and the base for research and academic freedom.

As a sincere Christian, personally involved in Church activities, John Mills showed warmth, kindness, and an understanding toleration of dissenting views in others. His personality was most evident in the small-group setting—tutorials, seminar, graduation, scientific meeting—where his pleasure in wine, bridge, climbing, botany, conversation and intellectual challenge would become readily apparent. In the best traditions of British human physiologists, his personal example induced a host of volunteers into a succession of potentially hazardous procedures, which seemed to be cheerfully tolerated by the subjects—if not by the alarmed dependents.

His death, within a few days of a definite decision to retire at 65, seems the more cruel and premature; but in the mountains he loved and after the successful completion of a challenging climb, perhaps the sense of tragedy may be alleviated.

S. Thomas

C. W. Ottaway

Christopher Wyndham Ottaway, Emeritus Professor of Veterinary Science in the University of Bristol, died on 14 February 1978 at the age of 67. He had occupied the chair of Veterinary Anatomy in the University from 1949–73, his being the second professorial appointment from the start of the School of Veterinary Science.

He qualified at the Royal Veterinary College, London and returned to join the staff of the Anatomy Department there in 1934, becoming a Reader in 1943. In the same year he was awarded a Fellowship of the Royal College of Veterinary Surgeons for a thesis on *The anatomical closure of the foramen*

ovale in the equine and bovine heart: a comparative study with observations on the foetal and adult states.

In 1945 Christopher Ottaway moved with a Senior Wellcome Research Fellowship to work in the Department of Zoology of Cambridge University under the late Professor Sir James Gray. There, while a member of King's College, he was able to develop his great interest in the functional anatomy of locomotion in animals and in 1948 he was awarded a Ph.D for a thesis in this field with special reference to the fore-limb of the dog. In the same year he was appointed to a lectureship in the Department of Zoology.

During this period, after publication of the Loveday Committee's second report on Veterinary Education in Great Britain, this topic was under continual debate. Christopher Ottaway was an active protagonist in support of the Committee's conclusion that all veterinary education should be within universities, a not too popular view at that time, either in universities or the veterinary profession.

It was fitting, therefore, that he was an early recruit to the staff of one of the two completely new university-based schools that resulted once Loveday's recommendations were put into operation. His appointment to the chair of Veterinary Anatomy at Bristol in 1949 meant that he was deeply involved in the development of the school from the start but at the same time he was able to continue his work related to locomotion and movement in animals.

He contributed a chapter on 'The Mechanism of Movement' in Hammond's *Progress in the Physiology of Farm Animals* (Vol. II, Butterworths, London, 1955). In 1961 he used as his title 'The Anatomy of Motion' in giving the Share Jones Memorial Lecture of the Royal College of Veterinary Surgeons (*Vet. Rec.* 74, 279–285, 1962). He had for many years been an admirer of the 18th century painter George Stubbs and he collaborated with the late Professor James McCunn in providing a commentary for the 1965 edition of Stubb's 1776 work *The Anatomy of the Horse* (J. A. Allen & Co. Ltd., London).

Stimulated by his interest the Bristol group of veterinary anatomists, together with a succession of post-graduate researchers, have contributed widely to the knowledge of locomotion, movement and related fields, and the

work continues. In addition, many years of undergraduates at London, Cambridge and Bristol will remember Professor Ottaway with gratitude for his enthusiastic teaching.

M. R. McCrea

Sir Rickard Christophers

A strong link with the malarial past has been severed by the death on 19 February 1978 of Sir Samuel Rickard Christophers, CIE, OBE, MB, FRS, at the age of 104 years. He qualified in medicine in 1896 at the University of Liverpool—2 years before the School of Tropical Medicine had opened its doors, and about the time that Ross discovered the transmission of malaria by mosquitoes.

In the previous decade the malaria parasite had been found by Laveran while most of the greatest discoveries in tropical medicine were being made at the turn of the century. Thus Christophers began his career in a fertile period; he knew all the famous figures, including Manson, the Father of Tropical Medicine, and was himself to contribute fundamental knowledge to the subject. He lost no time in making his way into the tropics and in 1897 went to the upper reaches of the Amazon as the medical officer of the ship.

The following year he became a member of the Royal Society—Colonial Office Malaria Commission and with J. W. W. Stephens he investigated the disease first in Nyasaland, then in various parts of West Africa and finally in India. This work, among other results, clearly demonstrated the connection between malignant tertian malaria, the consumption of quinine and blackwater fever. Furthermore, in 1900, Christophers published his first descriptions of the anatomy and histology of mosquitoes, observations which 60 years later were to culminate in his *magnum opus* on *Aedes aegypti*. In the meantime, he was to carry out detailed work on other insects including sandflies and ticks.

India finally claimed him in 1902 when he joined the Indian Medical Service; he spent the next thirty years in establishing malariology as a special discipline. His little book *How to do a malaria survey* (Christophers, Sinton & Covell) became the constant companion for all subsequent workers; the simplicity of its contents does not disguise the profound knowledge on which it is based.

These were the years of his greatest research: he was an entomologist, protozoologist and sanitarian; moreover he knew the subcontinent in many other aspects, its geology, flora and fauna, paleontology and sociology, and

of course the major epidemic diseases. Probably his most important discoveries related to the mechanism of immunity in 'hyper-endemic' malaria, in which he demonstrated the state of 'immune infestation'—the concept which had been foreshadowed by Koch, and was developed subsequently by Swellengrebel, Sergent and Bagster Wilson. His observations on splenomegaly led him directly to the problem of kala azar and eventually to the rôle of the sandfly in the transmission of the infection (the final solution of which had to await the classical experiments of the nonogenarian Colonel H. E. Shortt, his loyal and younger brother officer in the Service).

Christophers contributed to veterinary research by describing the development of *Babesia canis* in the tick, and to zoology by tracing the cycle of haemogregarines of rodents in ticks and lice. Within his broad interests he included systematics both as an entomologist and as a protozoologist; he and Stephens were the first to describe the characteristic stippling of erythrocytes caused by *Plasmodium falciparum* (and wrongly called 'Maurer's clefts') and he played no small part in the final agreement regarding the correct name of this parasite.

On his return to England, Christophers was given a personal professorship in the field of malaria studies at the London School of Hygiene and Tropical Medicine, where he carried out fundamental work on the biochemistry of malaria parasites and trypanosomes with J. D. Fulton. During this time also he was helped by P. G. Shute, who was a life-long friend. In 1938 he was given accommodation in the Department of Zoology in Cambridge, where he continued his anatomical studies of mosquitoes. He left Cambridge in 1963 and spent his remaining years in Dorset. His numerous friends remained in contact with him and had the benefit of his advice almost to the end of his life. His centenary was celebrated at the Royal Entomological Society and at the Royal Society of Hygiene and Tropical Medicine.

P. C. C. Garnham

L. Jánossy

Lajos Jánossy, Professor of Physics at Eötvös University, Budapest, died on 2 March 1978, at the age of 66. He was well known for his work on cosmic rays. His output of scientific papers was prolific: indeed, in 1972 a collection of his published papers filled five volumes. In addition he wrote a number of standard texts on cosmic rays, and less well known books on

the evaluation of measurements and relativity. Some of his books were later translated into Bulgarian, Hungarian, Italian, Japanese and Russian.

Jánossy began his study of cosmic rays with Kolhörster in Berlin for his doctorate. He came to England in 1937 and from 1938-47 was a prominent member of Blackett's distinguished school of cosmic rays at Manchester University. There he did significant work on various types of showers, primary cosmic rays and on the origin of the cosmic-ray meson. On the theoretical side he was much influenced by Heitler, then at the Dublin Institute of Advanced Studies. Like many others Jánossy did not solve the problem of the meson but his study of meson showers led directly to the Manchester cloud chamber work of 1946-52 and the discovery of some of the best known K-mesons and hyperons by Rochester, Butler and their colleagues. His research papers revealed a physicist with a thorough grasp of theory and mathematical ability of a high order.

In 1947 he was elected to a senior professorship at the Dublin Institute of Advanced Studies but he resigned in 1950 and returned to Hungary to play an important rôle in post-war scientific and academic development. He again set up a cosmic ray research group which did notable work on the time variations of the muon component and on extensive air showers and maintained close contacts with similar groups in the U.K. His other researches included the experimental and theoretical study of photons (especially photon bunching, which paralleled the work of Hanbury-Brown and Twiss in the U.K.), and the theories of relativity and quantum mechanics on which he held somewhat unorthodox views.

In addition to his University work he was from 1956-70 Director of the Central Research Institute for Physics where he worked to encourage the Hungarian electronics and other science-based industries. He was Hungarian representative on a number of international bodies particularly those connected with atomic energy and a member of the Hungarian Academy of Sciences (Vice-President from 1958-73).

Jánossy was slightly built, and of pale complexion but he had fine brown eyes which shone from under a shock of unruly dark hair. Former colleagues and his many friends will miss his scientific originality, his questioning mind and his warm friendship.

He married in 1937, Leonie Kahn, and they had three sons and one daughter. After Leonie's death he married Alice Farkas, a medical doctor.
George D. Rochester